FUNCTION OF CIKA IN THE CYANOBACTERIAL CIRCADIAN SYSTEM: THE *PSEUDO*-RECEIVER DOMAIN OF CIKA REGULATES THE CIRCADIAN INPUT PATHWAY

A Dissertation

by

XIAOFAN ZHANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Microbiology

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Approved by:

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Committee Members, John E. Mullet
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Jin Xiong
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ABSTRACT

Function of CikA in the Cyanobacterial Circadian System: The *pseudo*-Receiver Domain of CikA Regulates the Circadian Input Pathway. (August 2006)

Xiaofan Zhang, B.S., East China Normal University;

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The circadian input kinase gene (*cikA*) was first identified from a *Tn5* mutant of *Synechococcus elongatus* PCC 7942. A *cikA null* strain shows a striking phenotype related to circadian gene regulation: all sampled loci show a shortened circadian period and reduced amplitude of oscillation and a failure to exhibit a wild-type resetting of the phase of the rhythm after an environmental signal. This global defect in response to the environment suggests a key role for CikA in the circadian input pathways. Bioinformatics results classify CikA as a divergent member of the bacteriophytochrome family, suggesting a role in light signal transduction. *In vitro* analysis previously showed that CikA is a *bona fide* histidine protein kinase (HPK), and its kinase activity is regulated by the presence of other domains. Its own pseudo-receiver (PsR) domain is not the cognate receiver domain of its kinase HPK domain, and its GAF domain does not likely bind a bilin chromophore as do photoreceptive phytochromes. Recent results suggested that CikA may function as a redox-sensor.

In this study, we examined the function of each domain of CikA using different mutant *cikA* alleles, and determined their phenotypes with respect to complementation of a null mutant and overexpression in both wild type and *cikA* null strains. All domains except the featureless N-terminus were required for CikA function. Overexpression of all mutant alleles that encoded the PsR domain, whether or not the HPK was functional, caused a dominant arrhythmia phenotype. In the absence of PsR, overexpressed variants did not cause arrhythmia, but affected the amplitude and period of oscillation. The results suggest a model in which the PsR domain regulates kinase activity and mediates interaction with other input pathway components to allow CikA to reach the correct cellular position to fulfill its function. Cellular localization assays showed CikA can interact with a complex and showed a polar localization pattern, whereas its variant without PsR showed uniform distribution in the cell.

In summary, CikA is an autoregulated kinase in which the PsR domain regulates activity of the HPK domain and also serves as an interaction module to lead the CikA to a specific cellular position.

DEDICATION

I dedicate this dissertation to my dear mother, Xiuying Sun.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Susan S. Golden, for providing me the opportunity to study molecular genetics of bacteria and her guidance throughout this research project. I would also like thank the members of my committee, Dr. John Mullet, Dr. Deborah Bell-Pedersen, and Dr. Jin Xiong for the critical reviewing of my proposal, research progress, and this manuscript. I also should say thanks to my collaborators, Mr. Guogang Dong, Dr. Yoonsang Cho, Dr. Tiyu Gao, and Dr. Ioannis Vakonakis (John). I thank all of the Golden labfolks for their companionship and helpful suggestions.

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CHAPTER I

INTRODUCTION

The revolution of the earth around the sun and its rotation on its axis cause periodic changes of environmental variables, especially with respect to light and temperature (Pittendrigh, 1981). Consonant with the extrinsic rhythmic environments, most living organisms have been found to possess internal rhythmic processes that keep the same pace with the external conditions. A circadian rhythm, as one of the representatives of internal biological clocks, has been considered as an adaptive feature for centuries. It was first described by observing the leaf movements of higher plants (Sweeney, 1999). Then, other physiological and behavioral rhythms in diverse organisms were reported and studied further, such as the daily production of asexual conidia spores of *Neurospora crassa*, or the eclosion of *Drosophila melanogaster* (Bell-Pedersen *et al.*, 2005; Hardin, 2005).

CIRCADIAN RHYTHMS

For a typical circadian rhythm, there are three diagnostic characteristics: (1) a free-running period of about 24 hours under constant conditions, which is direct evidence that the circadian rhythms shown by living organisms are endogenous instead of imposed by the rhythmic environment; (2) the ability of entrainment, meaning that the biological clock can be synchronized by cycles or

This dissertation follows the style of *Molecular Microbiology*.

pulses of the environmental cues; and (3) temperature compensation, meaning that the rhythm maintains a relatively constant circadian period within the physiological range of temperature variation for the organism, in which the biological clock is kept stable and accurate (Aschoff, 1978; Golden *et al.*, 1997). Until now, all groups of eukaryotes, ranging from fungi to human beings, have been reported to show circadian rhythmicity (Aschoff and Wever, 1976; Bell-Pedersen *et al.*, 2005). The cyanobacteria are the only prokaryotes for which circadian rhythms have been clearly documented and well studied during recent decades (Bell-Pedersen *et al.*, 2005). In 2004, the J. Xiong group reported that a purple bacterium, *Rhodobacter sphaeroides*, also shows rhythms of expression of a reporter gene when the cells are grown under aerobic conditions, and that these rhythms have a period of about 20.5 hours. However, the organism shows ultradian rhythmicity (more than once per day) under anaerobic conditions, and neither rhythm has been shown to fulfill all criteria to demonstrate control by a circadian clock. Genes that are homologous to the circadian central oscillator genes of cyanobacteria are also found in purple bacteria (Min *et al.*, 2005). This promising data suggests a potential to understand the origin and evolution of circadian rhythms.

Despite the complexity of circadian systems among different species, a simplified model is still used to classify components according to their functions. This model (Figure 1-1A) contains three constituents: input pathways, central oscillators, and output pathways. The central oscillators generate and maintain

rhythms with a period of about 24 hours. Environmental cues, mainly light and temperature, are detected and transmitted through the input pathways to synchronize the pacemaker to the external daily cycles. The output pathways couple the central oscillator to the expression of clock-regulated genes and other circadian behaviors. Several organisms, which have been chosen as model systems to study in other research areas, are now also used to study circadian systems. These examples include rodents, the plant *Arabidopsis thaliana,* the fungus *N. crassa,* the insect *D. melanogaster*, and some cyanobacteria. Current studies on these organisms have greatly increased our understanding of circadian mechanisms among different species. Circadian systems in all organisms have been shown to contain complicated networks. An auto-regulatory feedback loop localized at the core of this network is found to be a general mechanism for the central oscillators in different organisms, although the central oscillator components may be different among species (Figure 1-1B) (Bell-Pedersen *et al.*, 2005). Positive and negative elements involved in this loop can be affected by input pathway signals to allow entrainment of the clock to the environment. The temporal information is then transferred through the output pathways to regulate rhythmic gene expression and biological activities. The cyanobacterium *Synechococcus elongatus* is one of the model systems and much authoritative research has been done to effectively reveal the mechanism of the cyanobacterial circadian system.

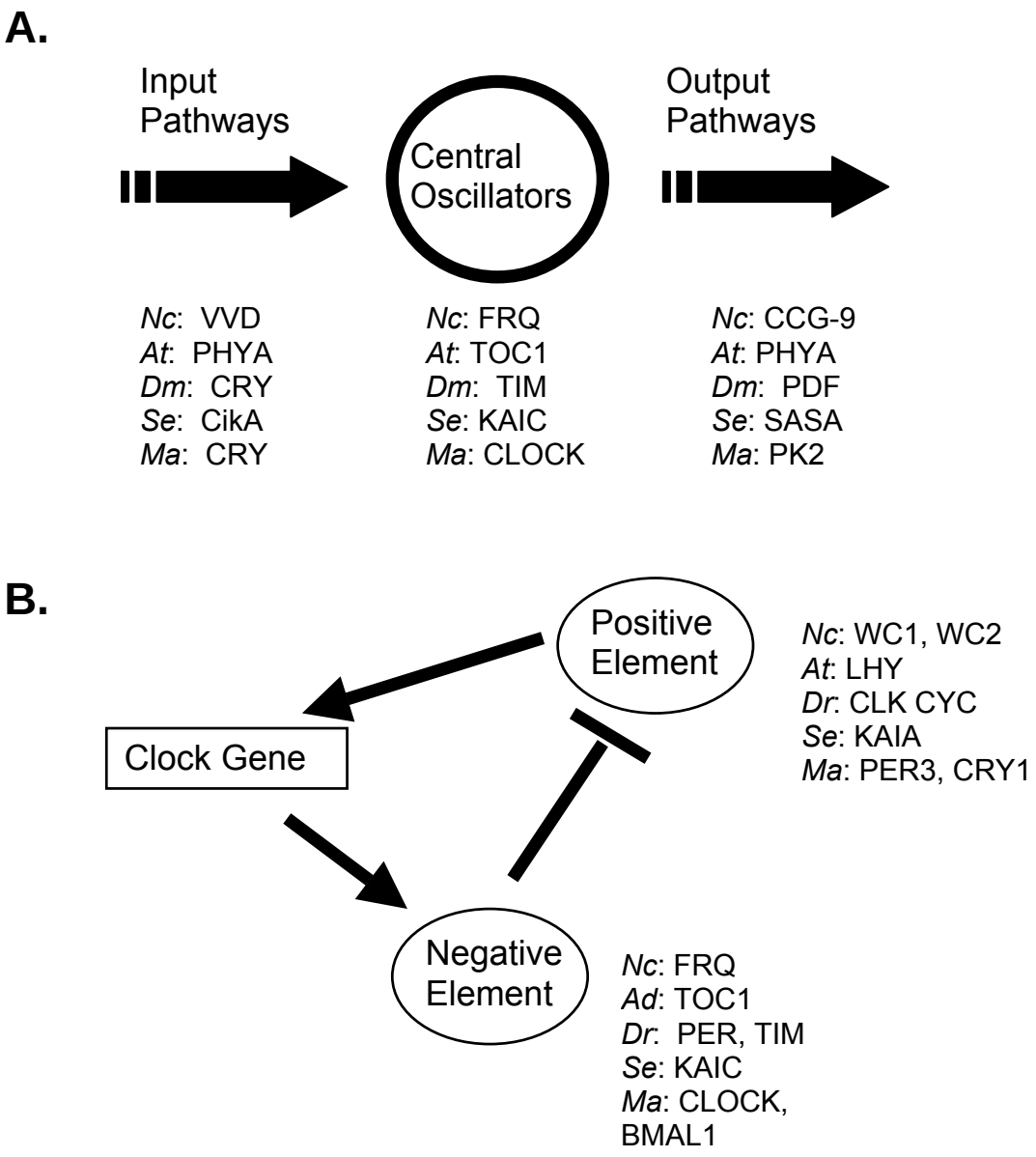


Figure 1. A simplified model of circadian systems and the negative-feedback loop model for the circadian oscillator.
(A) A simplified model of circadian systems. For each organism, only one representative of the clock components found in different organisms is listed.
(B) The negative-feedback loop model for the circadian oscillator that is thought to generally exist in different organisms. Some of positive or negative elements in different organisms are selected and shown in the figure. Adapted from Bell-Pedersen et al., 2005.
Nc—Neurospora crassa; At—Arabidopsis thaliana; Dm—Drosophila melanogaster; Se—Synechococcus elongatus; Ma—mammals.

SYNECHOCOCCUS ELONGATUS PCC 7942 IS AN EXCELLENT MODEL SYSTEM TO STUDY THE CIRCADIAN CLOCK

The original impetus that led to the discovery of the circadian rhythms in cyanobacteria was to clarify the diazotrophy-photosysnthesis paradox; how an organism can fix nitrogen in the same cells that generate oxygen during photosynthesis, because oxygen is extremely deleterious to nitrogenase (Johnson and Golden, 1999). Some filamentous cyanobacteria, like *Anabaena* sp. PCC 7120, can solve this problem through spatial separation of these two conflicting processes by cell differentiation to form two kinds of cells: vegetative cells for photosynthesis, and heterocysts for nitrogen-fixation, in which photosystem II (PSII) is turned off. How do the non-heterocyst filamentous species and the unicellular species resolve this conflict? The idea of temporal separation was brought to light from several different pieces of evidence. But It was not until 1985 that Stal & Krumbein published the first report which supported the existence of circadian rhythms of nitrogenase activity in a nonheterocystous *Oscillatoria* species (filamentous) (Stal, 1985). The report is considered as suggestive evidence of existence of circadian rhythms to temporally separate these two conflictive processes. Huang's group was actually the first to clearly recognize circadian rhythms of cyanobacteria. They demonstrated that all three salient properties of a circadian rhythm in the same organism, the unicellular freshwater species *Synechococcus* sp. RF-1, and were also the first to report the isolation of mutants affecting these circadian rhythms

(Chen *et al.*, 1996; Huang *et al.*, 1999). Further studies showed that other species of cyanobacteria also possess circadian rhythms, such as in the genera *Synechocystis* (Aoki *et al.*, 1995; Aoki *et al.*, 1997), *Cyanothece* (Schneegurt *et al.*, 1994), *Trichodesmium* (Chen *et al.*, 1998), and possibly *Prochlorococcus* (Shalapyonok *et al.*, 1998).

Currently, the model system used for circadian study is *S. elongatus* PCC 7942, previously known as *Anacystis nidulans* R2, which was the first cyanobacterium demonstrated to be reliably transformable by exogenously added DNA (Golden and Sherman, 1984; Golden *et al.*, 1997). Even though it cannot fix nitrogen, expression of genes for some processes shows similar circadian patterns to the circadian rhythms of nitrogenase. An example is a gene involved in purine synthesis, which was found to be inactive during the daytime and active at night (Liu *et al.*, 1996). Over decades, this organism has been used as a model system for multiple research areas including metabolism, photosynthesis, and response to environmental stresses (Golden, 1995; Johnson *et al.*, 1996). Many genetic tools have been developed. Compared with other circadian model systems, its genome is much small (only 2.7 Mb), even smaller than that of *Escherichia coli* (3.2 Mb). A small genome makes saturation mutagenesis to identify all the clock components feasible. In 2004, the genome was completely sequenced and is currently under annotation, which can facilitate gene identification and analysis (*http://genome.jgi-psf.org/finished_microbes/synel/synel.home.html*). In addition to these

advantages mentioned above, the development of bioluminescence reporter genes was most critical in developing this organism as an excellent model system to study circadian rhythms.

The luciferase gene *luc* from a firefly or *luxAB* from the marine bacteria *Vibrio harveyi* is fused to the promoter of a native gene, such as the promoter of a PSII gene (*psbAI*), and shows expression in *S. elongatus* as a function of activity of the promoter (Kondo *et al.*, 1993). The circadian patterns of bioluminescence provide phenotypes that are suitable for mutant identification (Figure 1-2). The bioluminescence can be automatically measured and recorded for several weeks using a specially designed machine (Figure 1-2). This technique efficiently facilitates mutant screening compared with manual sample collection methods. The bioluminescence pattern conforms to all three salient properties of circadian rhythms: persistence in continuous conditions (LL) with a period close to 24 h, entrainability by light/dark (LD) signals, and temperature compensation (Golden *et al.*, 1997; Kondo *et al.*, 1993). Under the control of the promoter of the *psbAI* gene, the luminescence rhythm shows the same pattern as *psbAI* gene expression, confirming the reliability of this technique for circadian study (Liu *et al.*, 1995a). In summary, these advantages have made *S. elongatus* the most developed cyanobacterial model system for circadian study.

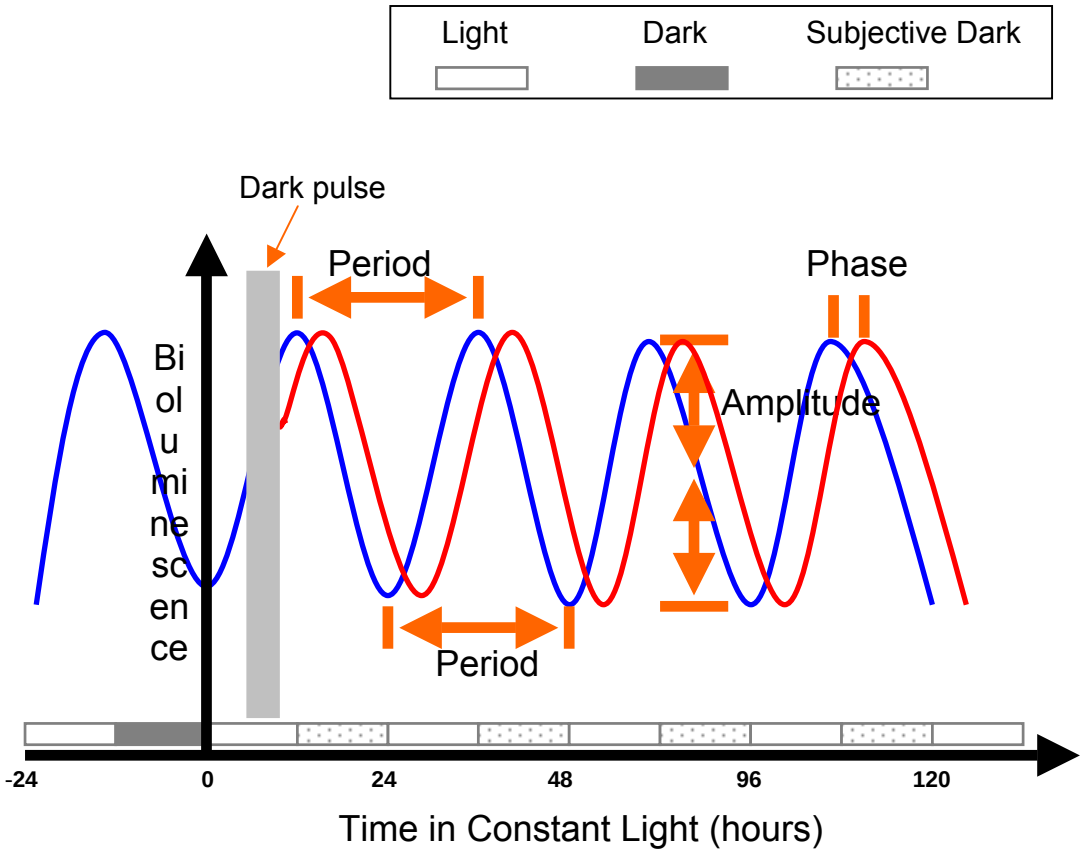


Figure 1-2 Circadian rhythms of bioluminescence in cyanobacteria and some fundamental circadian properties.
Strains containing luciferase reporter genes were synchronized by 12h light:12 dark before being exposed to constant light. Bioluminescence from the samples is measured at regular intervals and plotted *vs.* time. Period and amplitude can be measured according to the description in the figure. If a dark pulse is given at certain time point, the circadian trace (red) will be reset to a new phase but with same period and amplitude.
Period*:* circadian time from peak (or trough) to adjacent peak (or trough), and it is about 24 h.
Amplitude: Half the distance between peak and trough.
Phase: Instantaneous status of the circadian rhythm. This parameter is derived from the difference between reference points, which can be any points on the curve but must localize at the same position in different cycles. In the figure, the peak point is selected as the "reference point".

CURRENT CIRCADIAN MECHANISM MODEL FOR *S. ELONGATUS* PCC 7942

Taking advantage of the usage of bioluminescence reporter gene, biological clock mutants have been found, and corresponding genes have been identified and classified into different groups according to their function in the circadian system. Figure 1-3 summarizes much of the knowledge about the main circadian components and the relationships among them. Three oscillator genes, *kaiA, kaiB* and *kaiC* (*kai* means “cycle” in Japanese), were identified (Figure 1-3A); inactivation of any one of them or their combinations resulted in an arrhythmic phenotype (Ishiura *et al.*, 1998). For a long time, a timing loop of negative feedback by KaiC and positive feedback via KaiA on the promoter of *kaiBC* was thought to be the central oscillator because a similar mechanism is also found in other model systems, such as *Neurospora, Arabidopsis,* and *Drosophila* (Bell-Pedersen *et al.*, 2005) (Figure 1-1B). However, it was found in 2005 that a KaiC phosphorylation-dephosphorylation cycle, even without transcription and translation, is likely to be the central circadian oscillator. Self-sustainable oscillation of KaiC phosphorylation-dephosphorylation could be reconstituted *in vitro* by incubating KaiC with KaiA, KaiB, and adenosine triphosphate (ATP). The phosphorylation cycle shows a circadian rhythm and is also stable despite different temperatures of incubation (temperature compensation). The aforementioned negative feedback transcription-translation loop becomes an outer reinforcing layer over this core timing loop in the current model.

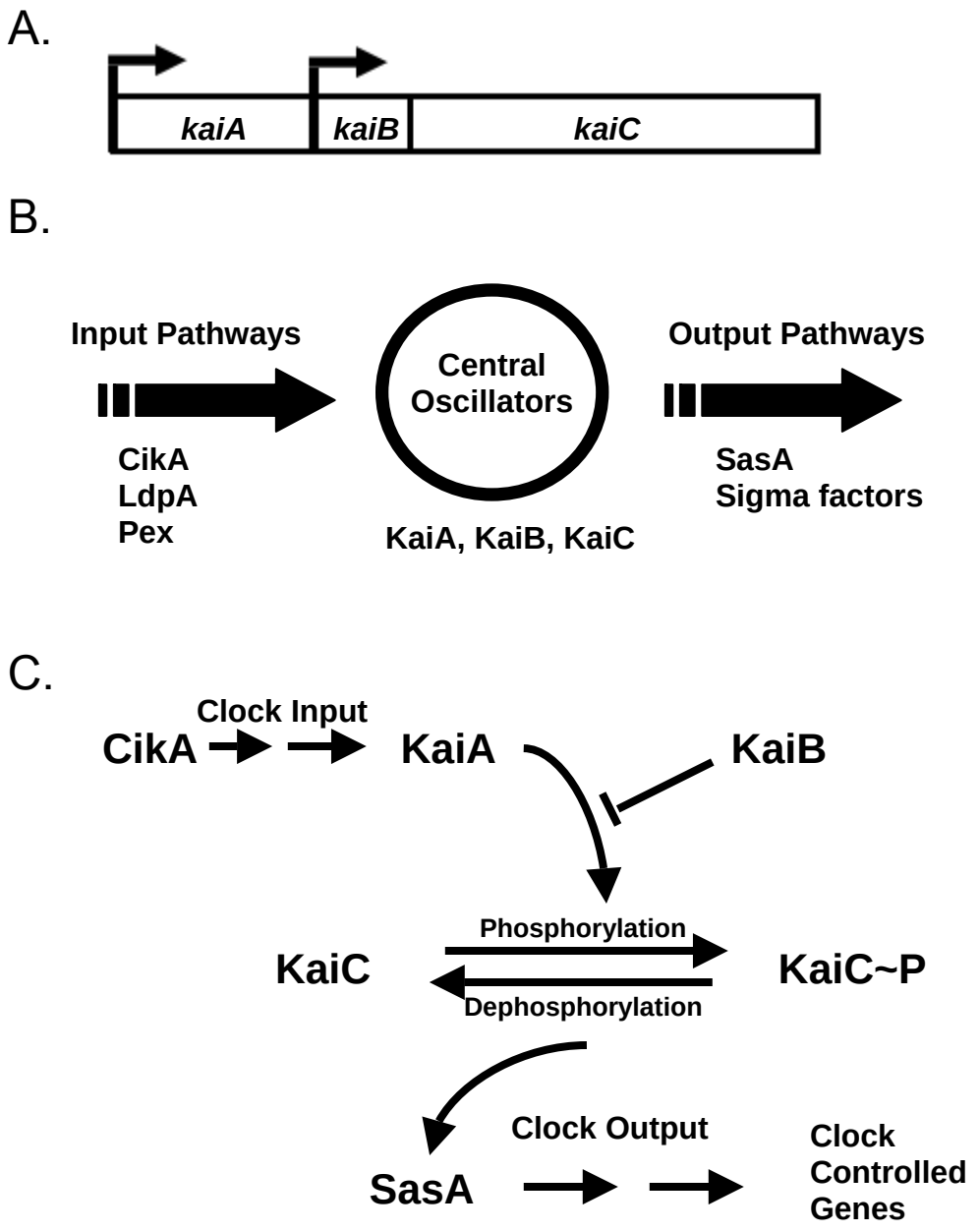


Figure 1-3 Current circadian mechanism model in cyanobacteria.

(A) Central oscillator genes: *kaiA, kaiB*, and *kaiC*. They are in the same gene cluster. KaiB and KaiC are dicistronic.

(B) A simplified model to show the main clock components that have been determined and classified according to their functions.

(C) Current circadian mechanism model in *S. elongatus*. Adapted from Golden SS *et al.*, 2004.

Using a promoterless *luxAB* randomly inserted into the genome of *S. elongatus,* all the bioluminescence-detectable colonies showed circadian rhythmicity that supported a global controlled pattern on gene expression in cyanobacteria. It suggests that all the genes in cyanobacteria appear to be clock-controlled genes (ccgs) (Liu *et al.*, 1995b). A critical output pathway component, SasA, was identified using the yeast two-hybrid system with KaiC as bait, which suggested a possible interaction between KaiC and SasA *in vivo*. Disruption of the *sasA* gene suppresses the expression of *kai* genes and all of other tested genes (Iwasaki *et al.*, 2000). The global suppression of rhythmicity in a *sasA*-null mutant and biochemical evidence suggests that the flow of information is from KaiC to SasA and SasA is most likely to be positioned at the root of the output pathway (Iwasaki *et al.*, 2000; Smith and Williams, Submitted for publication). Group 2 sigma factors (*rpoD2, rpoD3, rpoD4*, *sigC*) are also suggested to be involved in the regulation of ccgs (Nair *et al.*, 2002; Tsinoremas *et al.*, 1996). Nullifying some of them affects the rhythmicity of the expression of only a few genes instead of all of them. This suggests that the timing information from central oscillator involves multiple bransched output pathways to control subsets of genes, and suggests the possibility of the existence of multiple oscillators.

Environmental cues, such as light and temperature, are detected and transmitted through the input pathway components. CikA (**c**ircadian **i**nput **k**inase)

and LdpA (**l**ight **d**ependent **p**eriod) are determined to be involved in the input pathway. Knocking out the *cikA* gene will shorten the period of any strain by about 2 h regardless of its initial endogenous period (*e.g.,* in short- or long-period mutants). The *cikA* null strains also lose the ability to shift the phase of the rhythm in response to a 5 h dark pulse (Schmitz *et al.*, 2000) (Figure 1-2). These data support its role in the input pathway as a light detector. LdpA, an iron-sulfur protein, has been determined to be a gene involved in light-dependent modulation of the circadian period as a redox sensor. It can be co-purified with Kai proteins and CikA, suggesting its potential role to form a complex with Kai proteins. LdpA also affects the absolute level and light-dependent variation in abundance of CikA, another key input pathway component. These data suggest that LdpA is a novel input pathway component to the circadian oscillator that senses the redox state of a cell (Ivleva *et al.*, 2005; Katayama *et al.*, 2003).

PHOSPHORYLATION-DEPHOSPHORYLATION AND PROTEIN-PROTEIN INTERACTION ARE TWO MAIN POINTS OF REGULATION IN THE CIRCADIAN SYSTEM

According to the current circadian studies, phosphorylation-dephosphorylation and protein-protein interaction are demonstrated to be two important features in circadian mechanisms. For example, PERIOD (Per) and TIMELESS (Tim) in *Drosophila* can form a heterodimer through protein-protein

interaction to repress the expression of CLOCK and BMAL1, the other two central clock components that can also form heterodimers through protein-protein interaction (Hardin, 2000, 2004, 2005); FREQUENCY in *Neurospora* is degraded and/or translocated to the nucleus according to circadian rhythms of its phosphorylation state (Schafmeier *et al.*, 2006). These two mechanisms are also found to be functioning in the circadian system of *S. elongatus.*

At the post-translational level, phosphorylation and dephosphorylation of KaiC are now considered as the core timing loop. KaiA functions as an activator for the phosphorylation of KaiC, and KaiB functions as an attenuator of this activation process (Iwasaki *et al.*, 2002; Kitayama *et al.*, 2003; Nakajima *et al.*, 2005; Nishiwaki *et al.*, 2004; Tomita *et al.*, 2005; Williams *et al.*, 2002). Three phosphorylation sites in KaiC have been determined and mutating any one of them will block phosphorylation ability and abolish rhythmicity (Pattanayek *et al.*, 2004; Xu *et al.*, 2004). The input pathway component CikA and the output pathway component SasA, having been found to be two histidine kinases, can be phosphorylated and are assumed to be involved in two-component signal transduction systems. Two-component systems are common in bacteria to help them respond and adapt to changes in a variety of environmental conditions, including changes in osmolarity, nitrogen levels, and nutrients, and response to pathogens (Hoch, 2000; Stock *et al.*, 2000; Vierstra and Davis, 2000). A typical two-component system includes a histidine kinase and a response regulator. An input domain of the histidine kinase can detect a specific signal and stimulate

the autophosphorylation of the adjacent kinase domain. Then the phosphoryl group can be transferred to a partner response regulator protein at a conserved Asp residue, and its output domain will incite a response. The histidine kinase activity of CikA and SasA has been demonstrated to be necessary for their function, as their kinase-defective alleles cannot complement their respective null strains (Iwasaki *et al.*, 2000; Mutsuda *et al.*, 2003; Schmitz *et al.*, 2000). Their cognate response regulators are still unknown.

Kai proteins, CikA and SasA were also found to be able to interact with each other (Ivleva *et al.*, 2005; Iwasaki *et al.*, 1999; Kageyama *et al.*, 2003). Protein structure and biochemical characteristics of these clock proteins provide more details about their interactions. We currently know that KaiC can convert from a monomer to a hexamer in the presence of ATP (Pattanayek *et al.*, 2004). KaiA forms a dimer (Vakonakis and LiWang, 2004; Vakonakis *et al.*, 2004; Williams *et al.*, 2002; Ye *et al.*, 2004) and KaiB forms a tetramer (Iwase *et al.*, 2004). Results from the yeast two-hybrid system suggested that Kai proteins can interact with each other and are predicted to form heteromultimeric complexes (Iwasaki *et al.*, 1999). Copurification results verify this prediction, showing that KaiC forms protein complexes of approximately 350 kDa and 400-600 kDa *in vivo* during the subjective day and night, respectively (Kageyama *et al.*, 2003). This complex has been named the periodosome (Golden, 2004). In addition to the Kai proteins, SasA is also a periodosome component as determined using copurification. The size of the KaiC-containing complex oscillated with a

circadian rhythm, accompanied by nighttime-specific interaction of KaiA and KaiB with KaiC. Lack of any Kai protein or SasA will abolish the circadian rhythm in the formation of the clock protein complex, and also markedly affects the size of the protein complexes (Kageyama *et al.*, 2003). Thus, protein-protein interactions are a critical feature in the generation of circadian rhythm in cyanobacteria. Furthermore, LdpA copurifies with Kai proteins and CikA, which expands this feature to the input pathway (Ivleva *et al.*, 2005). The output pathway component SasA, of which the N terminus has a similar sequence as KaiB, also likely interacts with KaiC and is involved in the periodosome (Iwasaki *et al.*, 2000).

CIKA IS A KEY INPUT PATHWAY COMPONENT

In the cyanobacterium *S. elongatus* PCC 7942, the *cikA* gene, which was identified using random transposon mutagenesis, is considered to be a key component of the circadian input pathway, potentially as a light detector, based on the following phenotypes of *cikA* knockout strains (Fig. 1-4AB) (Schmitz *et al.*, 2000).

- Shortened period: Regardless of whether a wild type (WT) or period mutant background is tested, the circadian period for all tested genes is shortened by about 2 hours if *cikA* is nullified. This general defect in period is consistent with a role as an input pathway component.

- Low amplitude: compared with WT strains, *cikA* null mutants all show lower amplitude (half the distance from peak to trough) oscillations of gene expression for all genes tested.
- Loss of phase shifting ability in response to a 5 h dark pulse delivered at different time points during the circadian cycle; without CikA, light /dark signaling to the central oscillator seems to be blocked or weakened. However, a CikA mutant strain can still entrain to a 12 h light:12 h dark cycles (*i.e*., the cycle is lengthened to 24 h while the cells are in LD).
- Loss of phase shifting ability with a pulse of overexpression of KaiC. KaiC is an important central oscillator component, and its overexpression can reset the phase of the circadian rhythm (T. Kondo, personal communication). Thus, CikA also affects the transduction of feedback signals from the clock to the input pathway.

Obviously, CikA is not a central oscillator component because knocking out *cikA* does not cause an arrhythmic phenotype. However, without CikA, the circadian clock is hastened by about two hours, because the free-running period of all tested clock-controlled genes is shortened about two hours. This change suggests that CikA acts upstream of the central oscillator and directly affects the running of the clock. Without CikA, the transduction of light signals to the oscillator seems to be blocked or weakened. Thus, CikA is hypothesized to be a key input pathway component of the biological clock in *S. elongatus*.

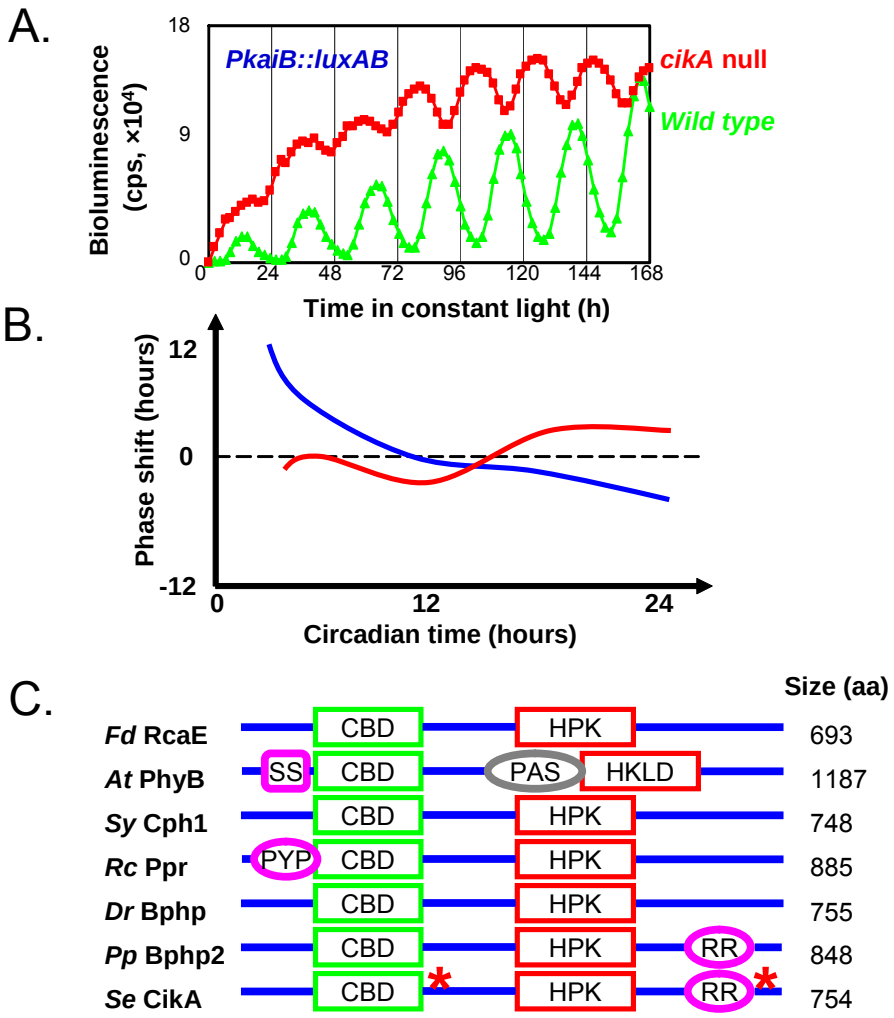


Figure 1-4 Phenotypes of *cikA* null and the phytochrome-like structure of CikA.

(A) Circadian phenotypes of WT and a *cikA* null strain. Green triangle—WT (period=25.0±0.4 h n=8); Red rectrangle— *cikA* null (period=23.1±0.1 n=12).

(B) Phase-resetting of the *PkaiB::luxAB* bioluminescence rhythm in WT (blue), *cikA* (red) genetic backgrounds in response to a 5-h dark pulse.

(C) Schematic diagram of phytochrome and bacteriophytochromes. (*Fd* RcaE -- *Fremyella diplosiphon* RcaE, *At* PhyB -- *Arabidopsis thaliana* phyB, *Sy* Cph1 – *Synechocystis sp*. PCC 6803 Cph1, *Rc* Ppr -- *Rhodospirillum centenum* Ppr, *Dr* BphP -- *Deinococcus radiodurans* BphP, *Pp* BphP2 -- *Pseudomonas putida* BphP2, *Se* CikA -- *Synechococcus elongatus* CikA. Domains: SS -- serine rich domain, PYP -- photoactive yellow protein domain, CBD-- chromophore binding domain (a subclass of GAF), HPK -- histidine kinase domain, HKLD--histidine kinase-like domain, PAS -- Per/Arndt/Sim repeats, RR--response regulator. Figure adapted from Vierstra RD 2000)

Bioinformatics analysis shows that CikA is a member of the extended bacteriophytochrome family (Figure 1-4C). These proteins are related to the plant photoreceptor phytochrome, which binds a bilin cofactor at its GAF domain (also called CBD) as a chromophore and has kinase activity that is regulated by red and far-red light. Large numbers of bacteriophytochromes have been discovered in widely diverse clades of both photosynthetic and non-photosynthetic prokaryotes, but for most of them, their physiological roles in environmental acclimation are still unknown (Montgomery and Lagarias, 2002; Vierstra and Davis, 2000). One member in this family is RcaE, a photoreceptor found in the filamentous cyanobacterium *Fremyella diplosiphon*; RcaE is involved in controlling complementary chromatic adaptation, a process that regulates the transcription of operons encoding light-harvesting proteins in cyanobacteria. Surprisingly, RcaE is a photoreceptor that is required for both green and red light responsiveness during CCA rather than red and far-red light like a common phytochrome in plants (Terauchi *et al.*, 2004). Thus, the wavelength of light that would be detected by CikA is still uncertain if CikA were to function as a phytochrome.

In addition to these circadian phenotypes, the *cikA* null strains also show a defect in cell division and the formation of FtsZ Z-ring is affected (Miyagishima *et al.*, 2005). Most of the cells of the null strain are longer than those of WT strains. Previous results showed that cell division is likely regulated by the circadian clock (Johnson *et al.*, 1996). A relatively short period of time during the

subjective night time was found that cell division is disallowed. These data tend to the prediction that CikA functions as an intermediate component to couple the circadian system with cell division.

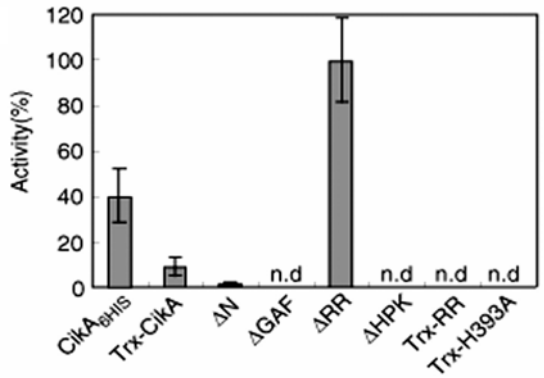
BIOCHEMICAL CHARACTERISTICS OF CIKA

In vitro studies had shown that CikA is an unusual phytochrome-like protein. According to amino acid sequence analysis, CikA possesses three distinct domains: a GAF, a histidine protein kinase (HPK), and a *pseudo*-receiver (PsR) domain (Figure 1-4C) (Schmitz *et al.*, 2000). GAF and HPK are commonly found in phytochrome-like proteins. However, the GAF of CikA lacks a conserved cysteine or histidine to serve as a bilin-binding site. Even though recombinant CikA from *E. coli* can bind an exogenous bilin compound like biliverdin or phycocyanobilin (PCB), CikA purified from the cyanobacterium is negative in a Zinc-fluorescence assay that tests for the presence of an attached bilin, leaving the nature of a potential *in vivo* cofactor still mysterious (Mutsuda *et al.*, 2003). *In vitro* autophosphorylation assays established that CikA is a *bona fide* kinase. If the conserved histidine (His) in the HPK at site 393 (residue 393 of the WT CikA ORF, GenBank accession AAF82192) is replaced with an alanine (Ala), the kinase activity of CikA is abolished. Phosphoryl transfer assays showed that the PsR domain cannot receive a phosphoryl from HPK, which suggests that PsR is not likely the cognate receiver domain of HPK. Thus, the

cognate receiver (named CikR) of CikA, if it exists, is still unknown (Mutsuda *et al.*, 2003; Schmitz *et al.*, 2000).

Another important property about CikA from the *in vitro* data is the regulation of its kinase activity by the presence of other domains. The kinase activity was examined for different CikA variants that resulted from truncations or point mutations [Figure 1-5A]. Because the truncated CikA variants are insoluble *in vitro*, a thioredoxin (Trx) was tagged to all versions of CikA variants as was a 6-His tag for purification purposes. Even though the kinase activity of Trx-tagged CikA is reduced compared with WT CikA, it still can complement a *cikA* null strain. Thus, the Trx-tagged variants can be studied with confidence of biological relevance and the results are shown in Figure 1-5A. As expected, mutation of the predicted H-box (His to Ala) eliminates autophosphorylation of CikA. The kinase activity of a variant without GAF cannot be detected, even though it contains an intact HPK domain. Deletion of the N terminus results in an extremely weak autophosphorylation signal although it retains all recognizable motifs of the protein. Surprisingly, removal of the receiver-like PsR domain enhances the auto-phosphorylation activity by more than 10-fold over the Trx-CikA full-length control. Based on these assays, a model was proposed in which the N-terminal and GAF regions positively regulate kinase activity, whereas the receiver-like domain suppresses it (Figure 1-5B) (Mutsuda *et al.*, 2003). For EnvZ, a histidine kinase of the same class in *E. coli*, the cognate response regulator OmpR can interact with the HPK domain to receive the phosphoryl

A.



B.

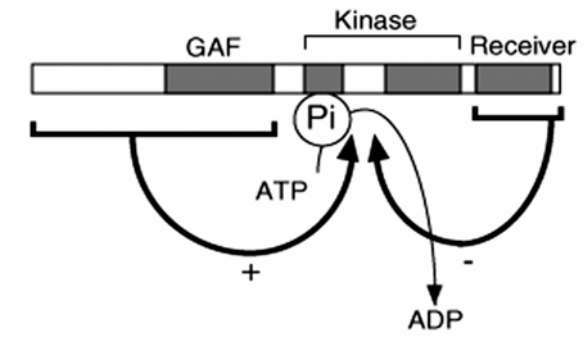


Figure 1-5 Autophosphorylation activity of HPK is regulated by the presence of other domains.

(A) Graphical representation of relative autophosphorylation activity. 100% is the average of the highest activity (n=4, ΔRR), n.d., not detectable. The abscissa shows different CikA variants, and the ordinate is the relative activity. CikA6His: 6His-tagged CikA; Trx-CikA: thioredoxin-tagged CikA; ΔN: thioredoxin-tagged CikA without N terminus; ΔG: thioredoxin-tagged CikA without GAF domain; ΔRR: thioredoxin-tagged CikA without *pseudo*-receiver domain; ΔHPK: thioredoxin-tagged CikA without HPK domain; Trx-RR: thioredoxin-tagged *pseudo*-receiver domain only; Trx-H393A: thioredoxin-tagged CikA with a point mutation at site 393, histidine→alanine.

(B) A model for the regulation of the kinase activity of HPK. Phosphorylation at the H-box of the kinase domain is regulated positively by the presence of GAF and the N-terminus and negatively by the *pseudo*-receiver.

(*Adapted from Mutsuda et al., 2003*)

group (Cai and Inouye, 2002; Mattison and Kenney, 2002; Yoshida *et al.*, 2002). We predict that PsR can interact with HPK and suppress the activity of HPK because its homologue to a *bona fide* receiver domain of response regulator.

CIKA MAY BE INVOLVED IN A PROTEIN COMPLEX

When LdpA, another important input pathway component, was used to pull down its interaction partners, CikA and Kai proteins were present in the product (Ivleva *et al.*, 2005). However, yeast two-hybrid system results do not appear to support the direct interaction between CikA and Kai proteins. Thus, it is possible that LdpA or other proteins may serve as intermediates to make up one big protein complex that contains input and oscillator components. In addition to LdpA, five interaction partner candidates of CikA were identified by yeast two-hybrid assays using CikA or PsR-containing CikA variants as bait (S.R. Mackey, J.-S. Choi, and Susan S. Golden, manuscript in preparation). These proteins may also function as intermediates between CikA and Kai proteins. Knock-out mutants for some of these candidates cause the changes in the circadian rhythm, even though none is similar to the phenotype of *cikA* null strains. Thus, it is possible that CikA is involved in the circadian machinery by protein-protein interaction.

OBJECTIVES OF THIS DISSERTATION PROJECT

Despite the abundant knowledge on CikA *in vitro*, fundamental questions regarding the function of CikA *in vivo* remain unanswered. This dissertation project is designed to investigate how CikA is involved in the circadian input pathway of *S. elongatus*. The approach focuses on the functional analysis of each domain of CikA. Structural information and cellular localization of CikA also provide important information for understanding the function of CikA. The specific aims of current research are: 1) to determine the function of each domain by examination of its requirement for complementation of *cikA* null strains and its contribution to overexpression phenotypes in both WT and *cikA* null strains for each CikA variant; 2) to examine the ability of each CikA variant to restore a phase shift to a 5-hour dark pulse and/or temperature pulse in a *cikA* null background; 3) to crystallize CikA and determine its structure by X-ray diffraction; and 4) to examine the cellular localization of CikA variants and their effects on cell division.

The next two chapters represent the results associated with this dissertation project. Chapter II is a manuscript that has been published in *Molecular Microbiology*, which describes the functional analysis of CikA *in vivo*. CikA was found to be a kinase that is autoregulated by its PsR domain. PsR regulates the kinase activity of CikA *in vivo* as suggested by *in vitro* assay of variants, the interaction of CikA with its interaction partners, and, as shown here, by changes in circadian parameters that correlate with the presence or absence of the PsR

domain in CikA variants. The cellular localization of CikA shows a focus at the cell pole that is dependent upon the presence of PsR. Chapter III describes the structural analysis of CikA, protein expression status of CikA variants *in vivo*, and the phase-shifting ability of CikA variants in response to temperature cues.

CHAPTER II

THE *PSEUDO*-RECEIVER DOMAIN OF CIKA REGULATES THE CYANOBACTERIAL CIRCADIAN INPUT PATHWAY*

INTRODUCTION

An endogenous biological circadian clock allows diverse organisms to adapt to their environment by anticipating predictable changes that are driven by the rotation of the Earth (Bell-Pedersen *et al.*, 2005; Edmunds, 1988). Environmental cues can be detected, interpreted, and transferred to entrain or reset the central oscillator of the biological clock through signal-transducing input pathways (Pittendrigh, 1981). Light is a potent time cue for the circadian clock, and photoreceptors have been classified as input pathway components in animals, plants, and fungi (Devlin, 2002).

A histidine protein kinase called CikA (**c**ircadian **i**nput **k**inase) is a key component of the circadian clock input pathway in the cyanobacterium *Synechococcus elongatus* PCC 7942, which is the simplest organism, in terms of genome size and unicellular structure, known to possess a canonical circadian biological clock (Ditty *et al.*, 2003). The *cikA* gene was first identified from a Tn*5* transposon insertion mutant that showed a subtle defect in light-responsive regulation of photosynthesis genes; subsequently, the mutation was

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shown to have a more striking effect on circadian rhythms of gene expression (Schmitz *et al.*, 2000). A *cikA* null strain still exhibits circadian rhythmicity as monitored by bioluminescence produced from luciferase reporter genes; however, the circadian period is shortened by about 2 hours, the amplitude of oscillation is greatly reduced, and, diagnostic of an input pathway defect, the ability to sense a 5-hour pulse of darkness that resets the phase of circadian rhythms in the wild-type (WT) strain is almost completely abolished (Schmitz *et al.*, 2000). Other factors that affect environmental sensing by the clock have been identified (Ivleva *et al.*, 2005; Kutsuna *et al.*, 1998), but none has as great an effect as CikA on the ability to reset the clock other than a component of the circadian oscillator itself (Kiyohara *et al.*, 2005); the oscillator is presumably the target of the input pathway (Williams *et al.*, 2002).

The proteins of the cyanobacterial circadian oscillator, KaiA, KaiB, and KaiC, have been identified and well studied at the genetic, biochemical, and structural levels (Golden, 2004; Iwasaki and Kondo, 2004; Nakajima *et al.*, 2005). Protein-protein interactions and the phosphorylation status of KaiC are two main principles of the central oscillator mechanism, and a circadian rhythm of KaiC phosphorylation can be established *in vitro* in a mixture of only KaiA, KaiB, KaiC, and ATP (Nakajima *et al.*, 2005). In the cyanobacterial cell, the three oscillator components daily form a large complex, together with other components, that has been named the periodosome (Iwasaki and Kondo, 2004; Kageyama *et al.*, 2003). The instantaneous composition of the periodosome may define particular

circadian times for transmission of temporal information to downstream processes.

Co-purification of CikA and Kai proteins with LdpA, another input pathway component, suggests that the input pathway is physically associated with the periodosome (Ivleva *et al.*, 2005). LdpA affects both light-dependent abundance of CikA and the sensitivity of CikA to cellular redox state. The requirement of LdpA for modulation of CikA abundance according to light intensity may be sufficient to explain the insensitivity of circadian period to light intensity that is observed in an *ldpA* null mutant.

A yeast two-hybrid assay does not detect direct interaction between CikA and LdpA, or between either of them and any individual Kai protein (Ivleva *et al.*, 2005). Thus, it is expected that intermediates are involved in the transfer of information from CikA to the Kai oscillator (Golden, 2003). Strains that lack *cikA* also exhibit a defect in cell division in addition to circadian phenotypes (Miyagishima *et al.*, 2005). Taken together, the data suggest a key role for CikA in bridging the circadian clock to fundamental cellular and metabolic activities, and suggest that CikA interacts with a network of partners.

Bioinformatics results predict that CikA contains three domains: a histidine protein kinase (HPK), a GAF (GAF) and a *pseudo*-receiver (PsR). CikA belongs to an extended bacteriophytochrome family (Ditty *et al.*, 2003; Montgomery and Lagarias, 2002; Vierstra and Davis, 2000), in which all of the proteins are homologous to plant photoreceptor phytochromes that typically bind a bilin

cofactor at their GAF domains and are light-dependent kinases. However, the GAF of CikA lacks a conserved cysteine or histidine to serve as a bilin-binding site. Moreover, CikA purified from the cyanobacterium is negative in a Zinc-fluorescence assay that tests for the presence of an attached bilin, leaving the nature of a potential *in vivo* cofactor still mysterious (Mutsuda *et al.*, 2003). *In vitro* autophosphorylation assays established that CikA is a *bona fide* kinase and demonstrated that the presence of other domains regulates its kinase activity (Mutsuda *et al.*, 2003). CikA variants have decreased kinase activity in the absence of the featureless 183-aa N-terminal segment, and non-detectable kinase activity without the GAF or HPK, or with a point mutation in the catalytic histidine of the HPK. Conversely, a 10-fold increased kinase activity when the PsR domain is removed reveals PsR as a negative regulator of kinase activity (Mutsuda *et al.*, 2003). Because two-component signal transduction systems are common in bacteria (Stock *et al.*, 2000), and CikA is a canonical HPK, it likely has a cognate response regulator (RR), a hypothetical partner termed CikR. The PsR domain of CikA cannot be phosphorylated by CikA HPK activity, consistent with absence from the PsR of the conserved aspartic acid present in the *bona fide* RR proteins that receive a phosphoryl group from an HPK (Mutsuda *et al.*, 2003); thus, the PsR domain is not CikR. PsR domains may function like the receivers of RRs in regulating an adjacent domain, but use protein-protein interactions rather than phosphorylation to effect conformational change (O'Hara *et al.*, 1999; Williams *et al.*, 2002).

In this study, we expressed variant *cikA* alleles to infer the function of each domain of CikA *in vivo*. The results showed that the GAF, HPK, and PsR domains are necessary for function of CikA, whereas the N-terminal featureless segment is expendable *in vivo*. Additional roles were revealed for the PsR domain, and led to a model in which the PsR domain interacts with other components near the cell pole, thus releasing negative regulation of the kinase after CikA reaches its proper position in the cell. In the absence of PsR, CikA cannot localize at its normal cell-pole position, as visualized by a green fluorescent reporter.

RESULTS AND DISCUSSION

Complementation ability of *cikA* mutant alleles

We examined the function of each domain of CikA through deletion analysis. A series of *cikA* in-frame alleles was constructed as listed in Table 2-1. All constructs were integrated at a neutral site (NS I) in the *S. elongatus* chromosome, and driven by a P*trc* promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). The P*trc* promoter exhibits low-level basal expression in the cyanobacterium (Mutsuda *et al.*, 2003), such that we could test both complementation in uninduced conditions, and an overexpression phenotype in the presence of inducer, for all constructs (Table 2-1). Circadian phenotypes were monitored by the oscillation in bioluminescence from a *kaiB::luxAB* reporter strain, which tracks circadian expression from the *kaiBC*

promoter. A *cikA* null strain exhibits a shortened circadian period (approximately 23 h rather than WT 25 h, Table 2-1 and Schmitz *et al.,* 2000), a reduced amplitude of oscillation (Figure 2-1C), a failure to reset the phase (relative timing of peak expression) after an environmental stimulus (Figure 2-2), and an elongated cell morphology .

In vitro studies have shown that truncated CikA variants are insoluble, and that a thioredoxin tag fused to their N terminus improves solubility and allows biochemical characterization (Mutsuda *et al.*, 2003). The thioredoxin tag, along with a 6-histidine tag to facilitate purification, is designated here as TH. As a first step for *in vivo* analysis, we determined that TH-CikA can complement the null strain even though, in an *in vitro* assay, its kinase activity is lower than CikA that carries only a 6-histidine purification tag (Mutsuda *et al.*, 2003). Normal circadian period and amplitude of *kaiB::luxAB* bioluminescence oscillation was restored to AMC1005, a *cikA* null reporter strain, by expression of TH-CikA (Table 2-1 and Figure 2-1E). Phase resetting ability (Figure 2-2) and normal cell size of the complemented strain (data not shown) confirmed that TH-CikA fulfills all known WT CikA functions. Thus, by comparing the complementation phenotypes of otherwise null *cikA* strains that carry ectopic alleles for TH-tagged CikA variants, it may be possible to infer the function of each domain. TH-CikA*, a missense

Table 2-1.Complementation and overexpression phenotypes of CikA variants

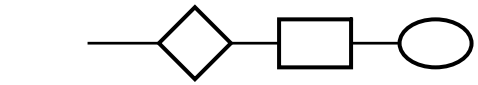
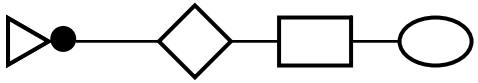
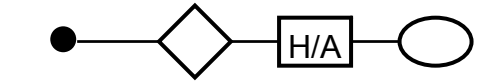
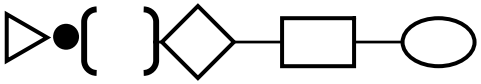
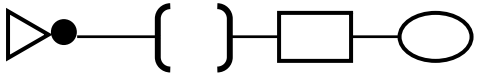
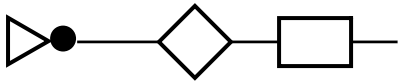
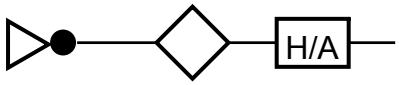
Name of variants	Architecture of Variant[1]	Complementation of *cikA* Null		Overexpression Phenotype[2]	
		Y/N	Period[3]	AMC1004	AMC1005
None	None		23.1 ± 0.1 (n=12)	WT	*cikA* null
WT[4]		Y	25.0 ± 0.4 (n=8) [4]	AR	AR
TH-CikA		Y	24.9 ± 0.1 (n= 8)	AR	AR
TH-CikA*		N	23.3 ± 0.1 (n= 9)	AR	AR
TH-CikAΔN		Y	24.8 ± 0.1 (n=12)	AR	AR
TH-CikAΔG		N	23.2 ± 0.1 (n=11)	AR	AR
TH-CikAΔP		N	23.4 ± 0.2 (n=11)	R[5], LA	*cikA* null
TH-CikA*-ΔP		N	23.4 ± 0.2 (n= 6)	R[5], MA	*cikA* null
H-PsR		N	23.6 ± 0.1 (n=12)	R[5], MA	*cikA* null

Table 2-1. Continued

[1] Symbols: line, CikA sequence; diamond, GAF domain; rectangle, HPK domain; rectangle with H/A, His to Ala substitution that renders HPK inactive; oval, PsR domain; closed circle, 6-histidine purification tag; triangle, thioredoxin solubility domain; brackets, deleted segment

[2] The variant indicated was induced by addition of 1 mM IPTG; background strain was either WT (AMC1004) or null (AMC1005) with respect to *cikA*.

[3] Circadian period expressed as average value in hours plus or minus standard error; n= number of samples assayed

[4] Previously published (Mutsuda, M., Michel, K.P., Zhang, X., Montgomery, B.L., and Golden, S.S. (2003). Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcus elongatus* PCC 7942. J Biol Chem *278*, 19102-19110).

[5] R, rhythmic (n=12 for each genotype) with a circadian period of: 23.5±0.1 (TH-CikAΔP); 23.8±0.1 (TH-CikA*-ΔP); 20.7±0.1 (H-PsR); LA, low amplitude compared with WT, equivalent to *cikA* null; MA, medium amplitude, higher than that of *cikA* null, but lower than WT.

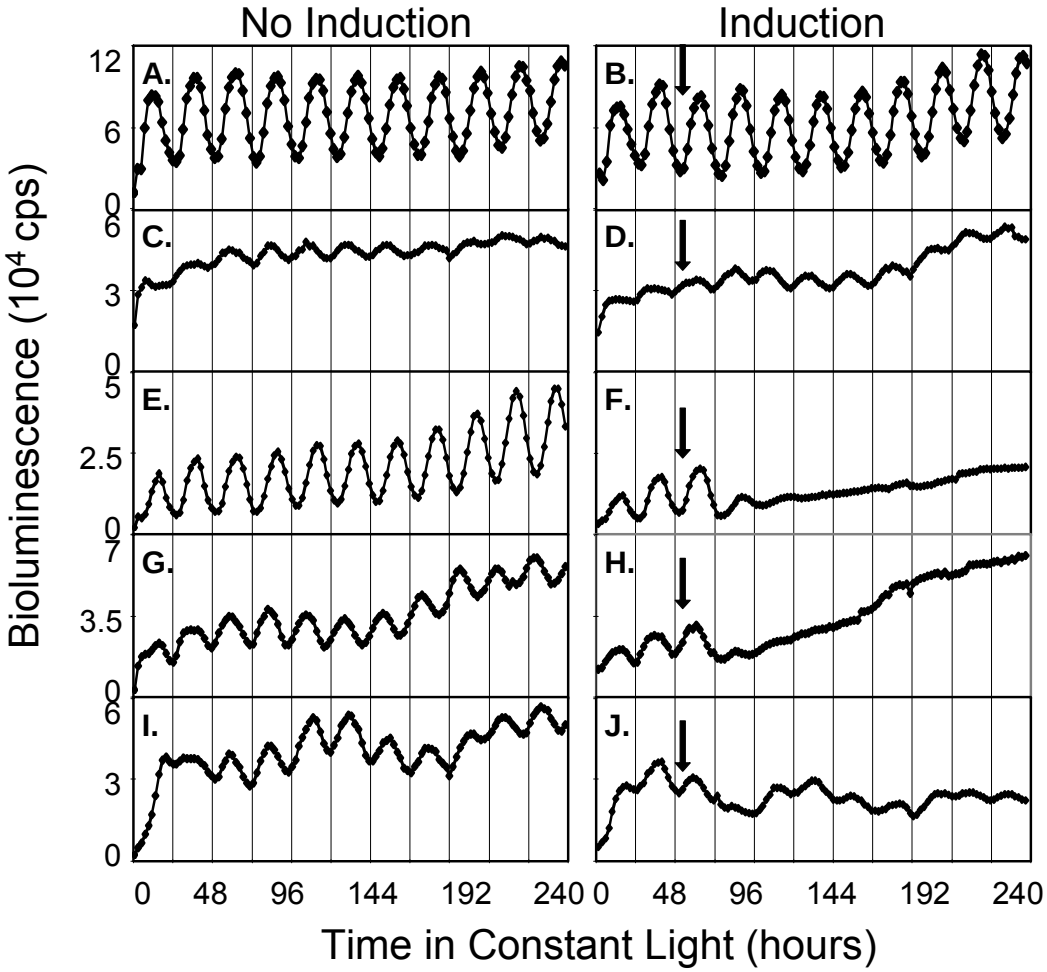


Figure 2-1 Representative circadian phenotypes of strains that carry CikA variants expressed in a *cikA* null background (AMC1005).

Strains were synchronized with two cycles of 12h L:12h D before exposure to constant light for monitoring. Bioluminescence was tracked by a Packard TopCount luminometer and expressed in counts per second (cps). Panels A, C, E, G, and I, no IPTG induction. Under these conditions, a fully-functional ectopic *cikA* allele is expressed at WT levels and complements the period and amplitude phenotypes of AMC1005. Panels B, D, F, H, and J, after addition of 1mM IPTG to induce high-level P*trc* expression at the time indicated by an arrow. Panels: A and B, AMC1004 WT control; C and D, AMC1005 (*cikA* null) control. All others are the AMC1005 background expressing the following variant: E and F, TH-CikA (and representative of an indistinguishable phenotype from TH-CikA?N); G and H, TH-CikA* (and representative of an indistinguishable phenotype from TH-CikA?G); I and J, TH-CikA?P (and representative of indistinguishable phenotypes from TH-CikA*?P, and H-PsR). Circadian periods, when oscillation was present, are reported in Table 2-1.

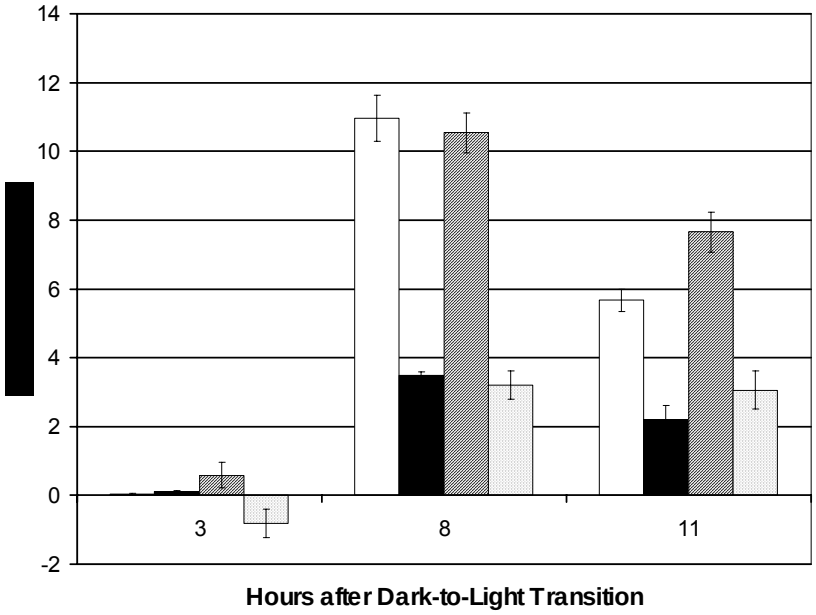


Figure 2-2 Phase-resetting phenotypes of WT and strains that express CikA variants in a *cikA* null background.
Phase resetting phenotypes of WT (AMC1004, open bars), *cikA* null (AMC1005, black bars), AMC1005 complemented by TH-CikA (hatched bars), and AMC1005 expressing TH-CikA* (dotted bars) in response to a 5-h pulse of darkness administered at different times during the circadian cycle. Samples were entrained to two light/dark cycles. The abscissa shows the time in hours after the last dark-to-light transition at which samples received 5 hours of dark incubation; after the dark pulse samples were returned to continuous light for monitoring of the circadian rhythm. The ordinate for each data point indicates the offset of the phase of peaks after the treatment relative to a control not pulsed with darkness: phase advance (positive values) or phase delay (negative values).

mutant at the His393 active site (residue 393 of the WT CikA ORF, GenBank accession AAF82192) in which kinase activity is abolished, failed the complementation test, confirming the requirement of kinase activity for CikA function *in vivo* (Table 2-1 and Figure 2-1G). The result supports the prediction that CikA signals are transferred through the type of two-component phospho-relay system that is common in diverse bacteria (Hoch, 2000; Stock *et al.*, 2000). The allele that encodes TH-CikAΔN, which lacks 183 aa at the N terminus, could complement the *cikA* null strain period and amplitude phenotypes (Table 2-1 and indistinguishable from Figure 2-1E) even though this variant has greatly reduced kinase activity *in vitro* compared to TH-CikA (Mutsuda *et al.*, 2003). Bioinformatics comparisons show no distinctive motifs in this expendable N-terminal region.

TH-CikAΔP, a variant that lacks the pseudo-receiver domain and which shows 10-fold stronger kinase activity than TH-CikA *in vitro* (Mutsuda *et al.*, 2003), could not complement the *cikA* null strain (Table 2-1 and Figure 2-1I). The failure of TH-CikAΔG to complement (Table 2-1 and indistinguishable from Figure 2-1G) may reflect loss of kinase activity, as suggested by previous *in vitro* data (Mutsuda *et al.*, 2003). Thus, PsR and GAF are required in addition to HPK, and, therefore, it is not surprising that TH-CikA*ΔP and H-PsR (both indistinguishable from Figure 2-1I) also failed the complementation test. In summary, functional CikA requires the presence of HPK, GAF, and PsR domains, and tolerates the absence of the 183-amino acid N-terminus.

Overexpression of PsR-containing CikA variants causes arrhythmia

Overexpression phenotypes of each *cikA* allele, in the presence or absence of a WT allele, may also help us infer function. The overexpressed protein may sequester partners in non-productive interactions, or reveal a partial function from a domain-deleted variant. Overexpression phenotypes were examined for each CikA variant in both the WT reporter strain AMC1004 (Table 2-1, Figure 2-3, right panels) and the *cikA* null strain AMC1005 (Table 2-1, Figure 2-1, right panels). After induction with 1 mM IPTG, overexpression of TH-CikA caused arrhythmia both in the null and WT backgrounds (Figures 2-1F and 2-3D). Possible mechanisms include excessive kinase activity after induction or non-functional sequestration of interacting components that are necessary for clock function.

Arrhythmia was also conferred by overexpression of TH-CikAΔN (indistinguishable from Figures 2-1F and 2-3D) or TH-CikAΔG (indistinguishable from Figures 2-1H and 2-3D), which have low kinase activity and no kinase activity *in vitro*, respectively, or of TH-CikA* (indistinguishable from Figures 2-1H and 2-3D), in which kinase activity is abolished. We conclude that kinase activity does not contribute to the dominant arrhythmic phenotype, and reasoned that sequestration of CikA-interacting partners is a likely explanation. The data suggest that CikA fulfills its normal function through interaction with other components in addition to likely phosphoryl relay through its HPK domain. Through which domain(s) does CikA interact with other components? The

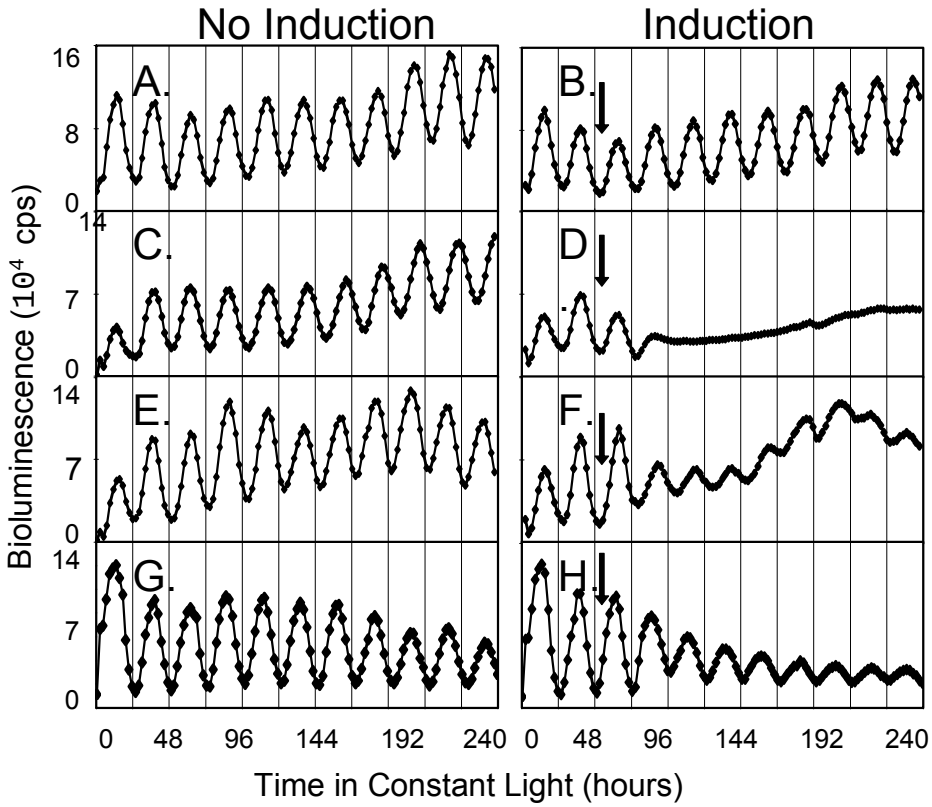


Figure 2-3 Representative circadian phenotypes of strains that carry CikA variants expressed in a WT background (AMC1004).

Experimental conditions were as for Figure 2-1. Panels: A, C, E, and G, no IPTG induction; B, D, F, and H, after addition of 1mM IPTG to induce high-level P*trc* expression at the time indicated by an arrow. Panels: A and B, AMC1004 WT control. All others are AMC1004 expressing the following CikA variant: C and D, TH-CikA (and representative of indistinguishable phenotypes from TH-CikA*, TH-CikA?N, or TH-CikA?G); E and F, TH-CikA?P; G and H, TH-CikA*?P. Axes are as in Figure 2-1; circadian periods, when oscillation was present, are reported in Table 2-1.

arrhythmic phenotype results from overexpression of TH-CikAΔN and TH-CikAΔG (indistinguishable from Figures 2-1F, 2-1H, and 2-3D), which excludes the N terminus or GAF alone as sole interaction modules unless the phenotype is a generic one that results from sequestering different sets of partners. Comparing the shared elements of TH-CikA, TH-CikA*, TH-CikAΔN, and TH-CikAΔG, we hypothesize that either HPK or PsR are required for the interaction that leads to arrhythmia upon overexpression. HPK domains are known to form homodimers through a specific subdomain, which is also the platform for interaction with its cognate RR (Dutta *et al.*, 1999). Furthermore, we confirmed that active CikA is a dimer in solution (see figure on pages 61 and 62). We reasoned that HPK primarily contributes to homotypic structure and phosphoryl transfer, whereas PsR may act as a module for interaction with other partners. Indeed, overexpression of a variant lacking PsR, TH-CikAΔP, failed to cause arrhythmia in either WT or null strains, but rather reduced amplitude and slightly shortened circadian period in WT strains (Table 2-1, Figure 2-3F). Thus, HPK is not sufficient, and PsR is necessary, to cause the dominant arrhythmic phenotype.

Overexpression of PsR alone is not sufficient to cause the arrhythmic phenotype

If PsR is the interaction module of CikA, the next question is whether overexpression of PsR alone causes a dominant arrhythmic phenotype as do

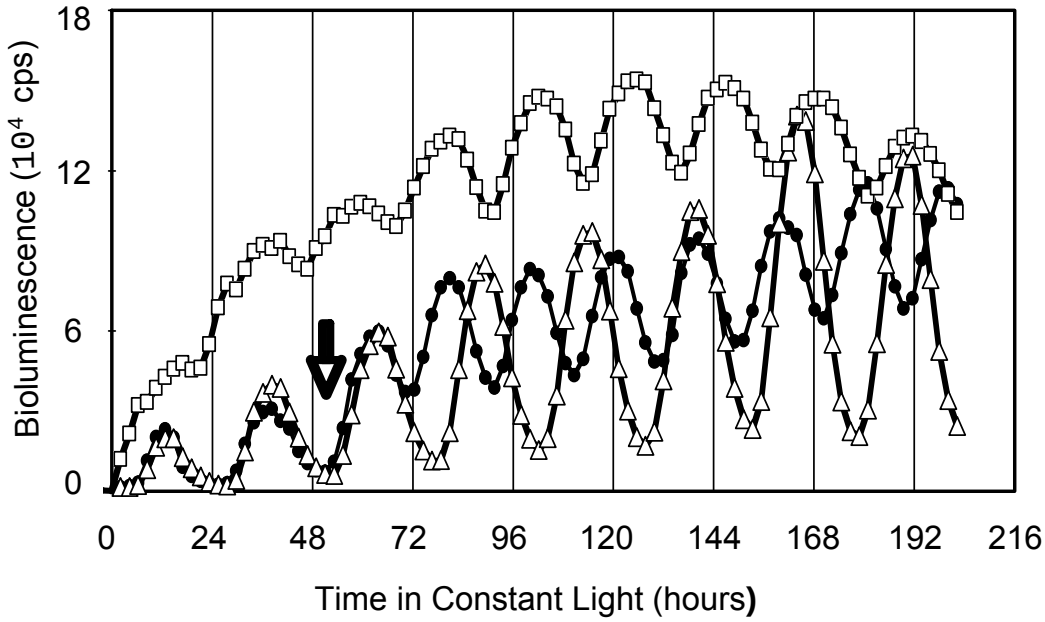


Figure 2-4 Circadian phenotype of H-PsR overexpressed in a WT background.
Circadian oscillation of bioluminescence from WT AMC1004 (triangles) compared with a *cikA* null strain (squares) and a strain that expresses H-PsR in a WT background (circles). At the time marked with an arrow 1 mM IPTG was added to induce expression of H-PsR. One cycle after induction the period changed from 24.7±0.1 h (WT 25.2±0.3) to 20.7±0.1 h, which is even shorter than the *cikA* null strain (22.9±0.08). Axes are as described for Figure 2-1.

other PsR-carrying CikA variants. We overexpressed H-PsR in both WT and *cikA* null strains (Figure 2-4), as H-PsR is known to be soluble *in vitro* without the thioredoxin tag (Gao *et al.*, 2005). The PsR overexpression phenotype is distinct from those of other PsR-containing variants: moderately reduced circadian amplitude and markedly shortened period (Table 2-1, Figures 2-4 and 2-5H). In both WT and *cikA* null backgrounds the strains maintain rhythmicity, indicating that PsR alone is not sufficient to cause the arrhythmic phenotype. Arrhythmicity may require both interaction of PsR with its partners and the sequestration of factors that interact with other parts of CikA. CikA is a dimer (see the figures on page 61 and 62) whereas isolated PsR is a monomer (Gao *et al.*, 2005). The difference in phenotype between overexpression of PsR and of other PsR-containing CikA variants may indicate that dimerization is needed for some partner interactions.

We propose that overexpression of PsR effectively blocks the CikA signal pathway: in a WT background, excess PsR occupies the binding site that is used by CikA to interact with downstream components such that CikA cannot dock into the correct position to complete signal transduction. If this is the case, it is not surprising that overexpression of PsR has no additional phenotype in a *cikA* null background (Figure 2-1J).

PsR negatively regulates the activity of HPK *in vivo*

TH-CikAΔP was overexpressed in a WT background (Table 2-1 and Figure 2-3F). A possible explanation for the resulting reduced amplitude and shortened

period is the increase in kinase activity that occurs when the negative-regulatory PsR is removed (Mutsuda *et al.*, 2003). Thus, overexpression of TH-CikA*ΔP, which lacks both the PsR domain and kinase activity, was tested in both WT and *cikA* null reporter strains. In the null background overexpression of this variant was indistinguishable from that of TH-CikAΔP (Figure 2-1J), causing no detectable change in the *cikA* null phenotype. Overexpression of either TH-CikAΔP or TH-CikA*ΔP in a WT background produced similar phenotypes, except that there is a notable difference in amplitude of the oscillation between the two mutant strains (Figure 2-3F and H). Because these variants differ only in kinase activity—expected to be higher than WT in TH-CikAΔP, and absent in TH-CikA*ΔP—the data suggest a specific link between CikA kinase activity and circadian amplitude. We reasoned that such a difference also should be distinguishable between full-length TH-CikA and TH-CikA* in the WT background under milder overexpression conditions than that which causes the arrhythmic phenotype. Immunoblot analysis revealed that after induction with 1 mM IPTG, constructs are expressed approximately 40 fold over the uninduced levels; the latter are approximately equivalent to WT CikA expressed from its native locus.

We performed a titration of induction of various constructs to further differentiate between kinase-proficient and -defective, and PsR-containing or -lacking, constructs by revealing quantitative changes in addition to fully-induced end-point differences. WT strains harboring TH-CikA and TH-CikA*, despite their

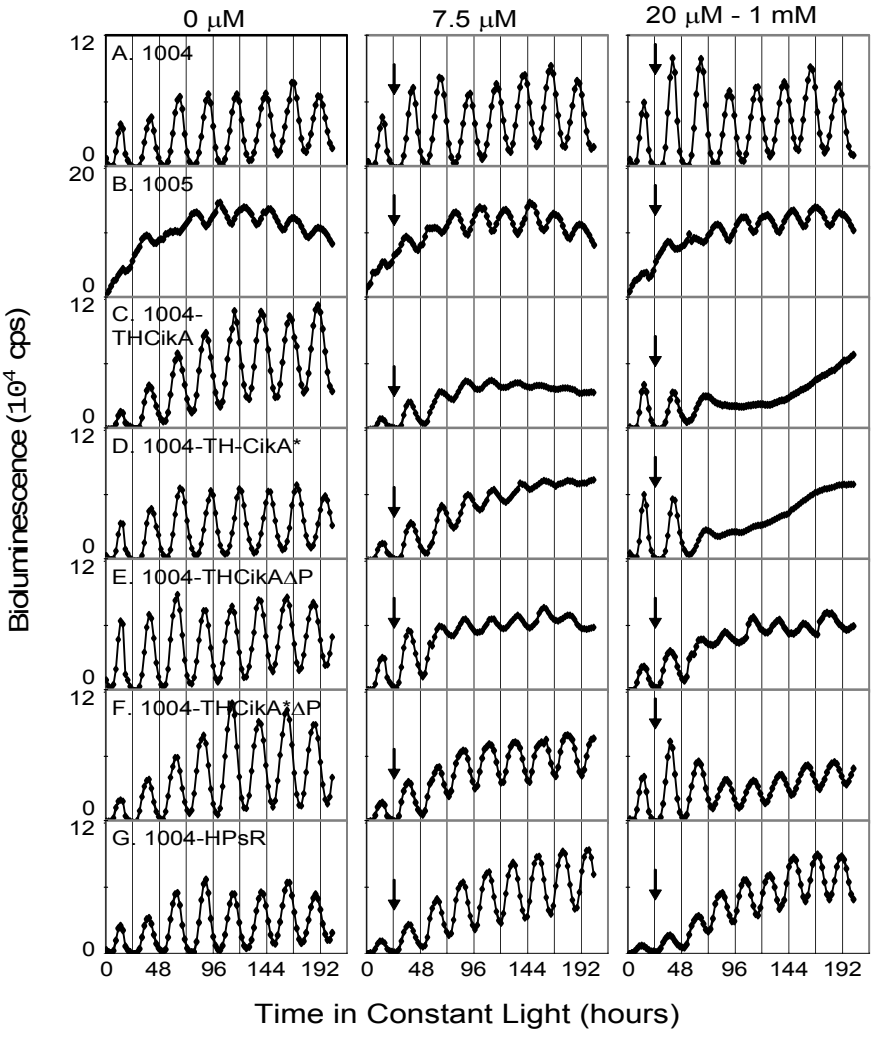


Figure 2-5 Changes in circadian phenotype after different levels of induction of CikA variants.

IPTG was added to samples at the following final concentrations (μM): 0, 2.5, 5, 7.5, 10, 20, 40, 60, and 1000. As a reference, TH-CikA and TH-CikA P are expressed at approximately 15-fold above WT CikA levels at 7.5 μM and 40-fold at concentrations above 20 μM IPTG. For the experiment in Panels A-G, representative traces are shown for 0, 7.5, and 20 μM IPTG added at the time indicated by an arrow, chosen as those concentrations that differentiated phenotypes among strains. A, AMC1004; B, AMC1005. Other panels show AMC1004 that expresses the variant indicated on the panel. All higher concentrations produced phenotypes indistinguishable from 20 μM. Axes are as for Figure 2-1. H, Circadian period of each strain after different levels of induction by IPTG. Circadian periods were calculated from the data depicted in panels A-G as well as from other concentrations of IPTG. Green filled triangle, AMC1004; blue filled triangle, AMC1005. AMC1004 expressing the following CikA variants: black filled circle, TH-CikA; red filled circle, TH-CikA*; blue open square, TH-CikA P; orange filled square, TH-CikA* P; green filled diamond, PsR. Above 10 μM induction no period could be calculated for TH-CikA and TH-CikA*, which produce arrhythmia when overexpressed. Panels show average values (n=12) with corresponding standard error.

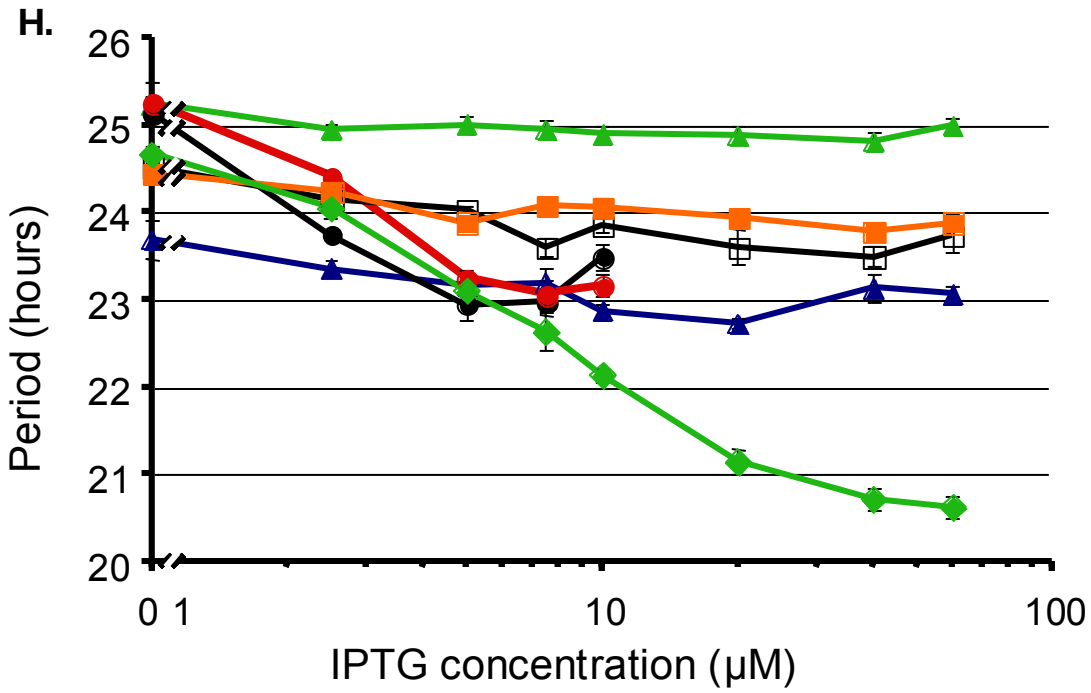


Figure 2-5 continued.

difference in kinase activity, showed a similar pattern in change of amplitude and period (Figure 2-5C, D and H) even at very low levels of IPTG (including 2.5 μM, not shown in Figure 2-5C and D). We concluded that overexpression of TH-CikA or TH-CikA* causes the dominant arrhythmic phenotype, and an intermediate phenotype at lower induction levels, through interaction with other components without evidence of contribution from kinase activity. This might be expected if the kinase activity of overexpressed TH-CikA is repressed in the full-length protein by PsR.

Overexpression of TH-CikAΔP and TH-CikA*ΔP, in which PsR repression of the kinase would not occur, produced different amplitude phenotypes at various levels of induction (Figure 2-5E and F). Both strains were reduced in amplitude relative to WT, but the amplitude phenotype was much less severe in the absence of kinase activity (Figure 2-5F) at any given level of induction. We propose that an effect of kinase activity on circadian amplitude is uncovered in the overexpression phenotypes of TH-CikAΔP and TH-CikA*ΔP, but masked in those of TH-CikA and TH-CikA*. The difference between these two groups is the presence of the negative regulatory PsR, consistent with *in vitro* kinase activity assays (Mutsuda *et al.*, 2003).

We also titrated the induction of H-PsR in a WT background to determine the influence of this domain alone (Figure 2-5G and H, Table 2-1). This mutant phenotype had a shorter circadian period than even the *cikA* null strain (Figures 2-4 and 2-5G and H), and an amplitude similar to that of TH-CikA*ΔP

overexpression. If our hypothesis is correct that the PsR domain is used to dock CikA with other partners, then excessive PsR may take the place of WT CikA in that complex; however, this should be a dynamic process in which PsR might not entirely exclude the docking (and kinase stimulation) of CikA. Thus, the phenotype might be expected to differ somewhat from a *cikA* null strain, in which kinase activity is completely absent. Because PsR is likely to have a similar domain structure to a *bona fide* receiver domain, and thus, similar to that of the unidentified cognate partner of CikA, overexpressed H-PsR may also compete in *trans* with the partner RR for interaction with the HPK domain of CikA. Genome sequence analysis revealed 18 RR proteins encoded by the genome of *S. elongatus*. Several of these loci encode candidates for CikR, and we are currently targeting these for analysis (S.R. Mackey, H. Malcolm, E. Clerico, and S.S. Golden, unpublished data).

PsR is required for CikA to localize at the pole

If PsR is responsible for interaction with partners, especially through a specific docking as we have proposed, we would predict that the localization of CikA in the cell would be different with and without the PsR domain. The commonly used Green Fluorescent Protein (GFP) from *Aequorea victoria*, which works well in other cyanobacteria (Wu *et al.*, 2004), does not fluoresce in *S. elongatus* although we can detect its expression immunologically (N.B. Ivleva and S.S. Golden, unpublished data). ZsGreen (ZsG), a green fluorescent protein from a *Zoanthus* sp. coral, was successfully used to determine the cellular

Figure 2-6 CikA shows polar localization but loses this pattern without PsR.

DIC images are shown for the WT (AMC1004, panel A) and *cikA* null (AMC1005, panel G) strains. Ectopically-expressed CikA variants that do (TH-CikA, panel C) or do not (TH-CikA? P, panel E) correct the mild cell division defect are shown. Fluorescence images are shown for AMC1004 and AMC1005 transformed with ectopically-expressed alleles that encode: unfused ZsG (panels B and H, respectively); AMC1005 expressing ZsG-TH-CikA (panel D) or ZsG-TH-CikA? P (panel F). Panels D and F are shown at slightly higher magnification to clarify distribution of the ZsG-CikA variants. In all cases, the scale bar represents 5 μm. Green fluorescence indicates the fusion protein; red fluorescence arises from autofluorescence of chlorophyll in the photosynthetic apparatus and marks the cell periphery where thylakoid membranes are located. The difference in cell length between panels D and F is meaningful, as the full-length ZsG-TH-CikA allele complements all phenotypes of *cikA*, including the cell division defect, whereas the PsR-truncated allele does not. Expression of the ZsG-encoding alleles was induced with 1 mM IPTG to facilitate imaging; the same localization was observed by eye without induction in each case.


A. AMC1004
B. AMC1004-ZsG
C. AMC1005-THCikA
D. AMC1005-ZsG-THCikA
E. AMC1005-THCikAΔP
F. AMC1005-ZsG-THCikAΔP
G. AMC1005
H. AMC1005-ZsG

localization of CikA in *S. elongatus*. Genes that encode ZsG-tagged TH-CikA and TH-CikAΔP were constructed and transferred into *S. elongatus*. The *zsg-TH-cikA* allele was able to complement the *cikA* null strain and its overexpression phenotype is the same as for TH-CikA; likewise, ZsG-TH-CikAΔP functions like TH-CikAΔP (data not shown). As shown in Figure 6B and H, unfused ZsG is globally distributed in the cell. ZsG-TH-CikA showed a polar localization pattern, with one or two foci per cell (Figure 2-6D). ZsG-TH-CikAΔP, in which the PsR domain is missing, showed a global distribution pattern similar to that of unfused ZsG (Figure 2-6F), which supports the hypothesis that PsR is an interaction module that is crucial for the proper localization of CikA.

The polar localization pattern of CikA is consistent with its function related with cell division (Miyagishima *et al.*, 2005). Cells of a *cikA* null strain are abnormally long, which suggests a defect in cell division without CikA (Figure 2-6E-H) (Miyagishima *et al.*, 2005). Several protein kinases related with cell division, such as PleC, DivJ and CckA, as well as some of their response regulators, localize at the poles in *Caulobacter crescentus*, a model system for cell division (Quardokus and Brun, 2003). CikA may similarly complex with proteins to regulate cell division. The recent identification of other loci involved in cell division may help to explain how these two major periodic processes, cell division and the circadian system, are interrelated in *S. elongatus*.

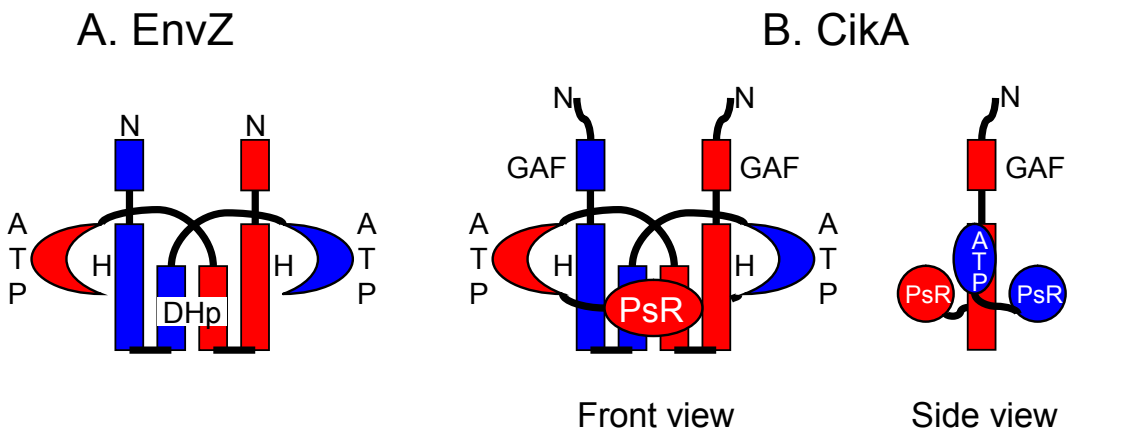
Model for function of CikA

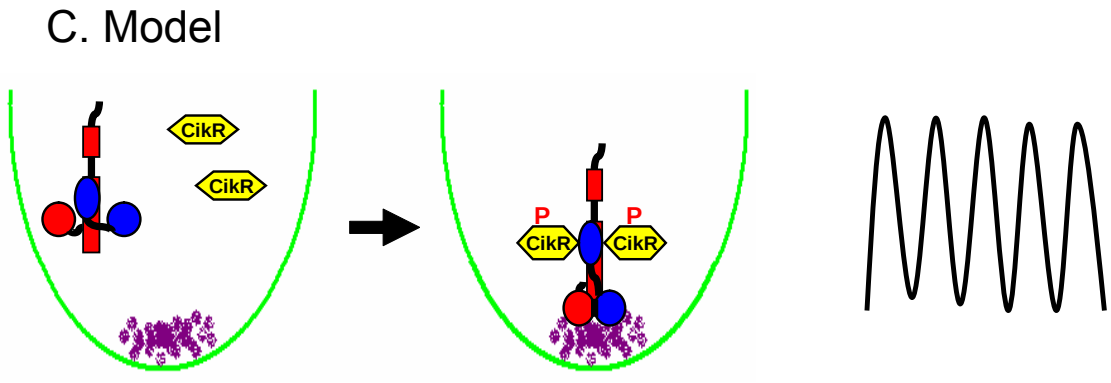
Amino acid sequence analysis showed the HPK domain of CikA to be related to the class I histidine kinases as exemplified by EnvZ, whose structure has been partially determined (Dutta *et al.*, 1999). Thus, the C-terminal portion of dimeric CikA (Figure 7B) is likely to have a structure similar to EnvZ (Figure 2-7A) as it relates to the kinase domain. The HPK can be divided into two sub-domains, a catalytic and ATP-binding domain, and a dimerization and histidine phosphotransfer domain (DHp), which are well conserved in CikA. Based on the structure of EnvZ and its interaction with its cognate RR, OmpR, we expect that the DHp domain of CikA is the interaction site for CikR to dock and receive a phosphoryl group from the catalytic H box. We propose that the receiver-like PsR domain also interacts with the DHp domain (Figure 2-7B) and blocks the interaction of CikA with CikR when CikA is not docked to its binding site (Figure 2-7C). This hypothesis is based on the negative regulation of kinase by PsR *in vitro*, the structural similarity of PsR to RR receivers as determined by NMR data, and inference of PsR influence on kinase activity from the data in Figure 2-5.

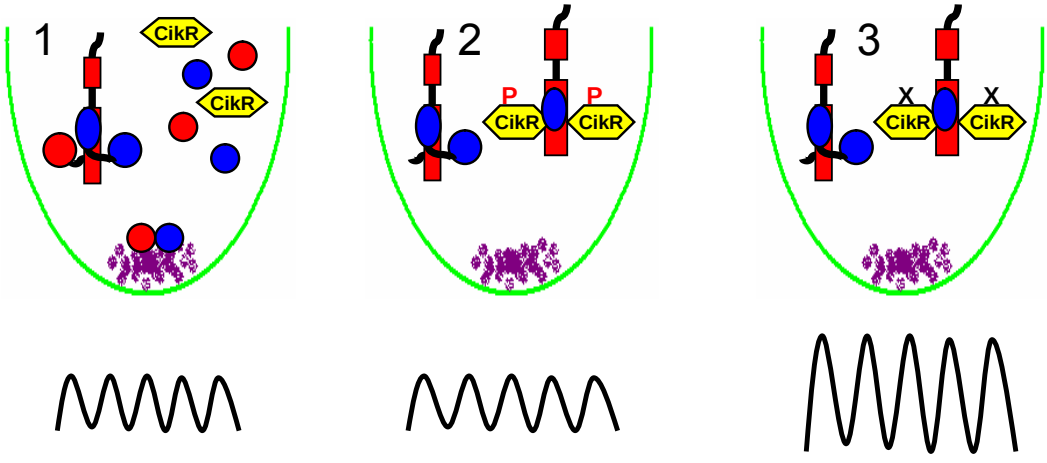
The aggregate genetic, biochemical, bioinformatic, structural, and cell biological data led to the following model. CikA transmits information in the circadian input pathway as an autoregulated kinase in which the PsR domain fulfills two important functions (Figure 2-7C): PsR (1) negatively regulates the kinase activity of HPK through intramolecular interaction, which also excludes binding of CikR at the DHp domain; and (2) interacts with a specific partner(s),

Figure 2-7 Inferred structure and model for CikA function.

(A), Representation of structure of EnvZ (adapted from Dutta R. *et al.*, 1999) and (B), inferred structure of CikA. For EnvZ, only the cytoplasmic catalytic region is represented. Each color depicts one monomer. (C), Model for the function of CikA. The side view from panel B is used for clarity. In the cytoplasm, PsR negatively regulates the activity of HPK by interaction with the DHp domain. Thus, the presumed cognate RR, CikR, cannot interact with DHp to receive a phosphoryl group from HPK. When CikA docks into its proper position at the poles through PsR, the HPK domain is exposed and interacts with CikR to complete phosphoryl transfer. CikA is thought to form a large complex with other proteins that are also localized at the poles. High amplitude WT circadian rhythmicity results. (D), Overexpression case 1: excess PsR in a WT background competes with CikA for binding to the dock and competes with CikR for binding to the DHp domain on CikA. Thus, only occasionally is signal transduction completed; circadian oscillation is low amplitude and has a very short period. Overexpression case 2: excess TH-CikA? P heterodimerizes with CikA. The heterodimers have a single PsR domain, and cannot dock at the polar binding site. Phosphorylation occurs in the cytoplasm, and circadian period and amplitude are affected. Overexpression case 3: excess TH-CikA*? P heterodimerizes with CikA and forms ineffective heterodimers that neither phosphorylate CikR nor dock at the pole; circadian period and amplitude are moderately affected.


A. EnvZ
B. CikA
N
N
A
T
P
H
H
DHp
A
T
P
N
N
GAF
GAF
A
T
P
H
H
PsR
A
T
P
N
GAF
A
T
P
PsR
PsR
Front view
Side view


C. Model
CikR
CikR
P
P
CikR
CikR


D. Overexpression
1
CikR
CikR
2
P
P
CikR
CikR
3
X
X
CikR
CikR

which localizes CikA at the pole and allows it to participate in a large complex with clock-related proteins and other unknown components. PsR interaction with its non-CikA partner at the pole releases inhibition of HPK by PsR; CikR can then dock into DHp and receive the phosphoryl group to activate transduction relay. Thus, PsR could serve to regulate not only when, but also where, phosphorelay takes place. Overexpressed PsR competes with CikA for the docking site, and with CikR for binding to the DHp (Figure 2-7D, case 1). The high kinase activity caused by removal of PsR in overexpressed TH-CikA∆P dramatically affects circadian amplitude in a WT background (Figure 2-7D, case 2), which may reflect CikR phosphorylation by CikA heterodimers in the cytosol instead of at the pole. Overexpressed TH-CikA*∆P, without kinase activity (Figure 2-7D, case 3), also somewhat reduces amplitude compared with the WT strain; in this scenario, overexpression likely ties up other factors which interact with the remainder of CikA, but does not cause arrhythmia when the heterodimers cannot dock.

GAF, as well as the DHp subdomain of HPK, may also contribute to CikA dimerization as do the GAF domains of an adenylyl cyclase whose structure has been solved (Martinez *et al.*, 2005; Qin *et al.*, 2000). Thus, the lack of complementation of TH-CikA∆G, and loss of kinase activity, may result from loss of dimerization activity, as natural HPKs are not known to function as monomers (Qin *et al.*, 2000). Alternatively, the importance of GAF may lie with its binding of an unidentified ligand and regulatory aspects associated with bound and

unbound changes in conformation of the protein, or through physical interaction with protein partners of its own.

It is likely that CikA interacts with proteins other than CikR and the proposed docking site/inhibition-releaser of PsR. Five potential interactors have been identified through a yeast 2-hybrid screen, of which two specifically recognize a PsR domain bait and three others require the presence of at least HPK (S. Mackey, J.-S. Choi, and S.S. Golden, manuscript in preparation). Whether either of the conserved hypothetical proteins that interact with PsR fulfills the functions proposed in the model is under investigation. Although CikA is a well-conserved HPK, and a partner CikR is predicted by precedent to exist, it is also possible that CikA works through autokinase and autophosphatase activities that are regulated by, and regulate, interactions with other partners without a transfer of the phosphoryl group to a receiver domain of another protein.

The importance of post-translational events in circadian regulation in *S. elongatus* was portrayed dramatically by the demonstration that a circadian rhythm of KaiC phosphorylation can occur *in vitro* when only KaiA, KaiB, KaiC, and ATP are present (Nakajima *et al.*, 2005). In the context of the cell, this core oscillation is integrated with other events that allow synchronization of the circadian oscillation (involving CikA) and relay of temporal information to clock-controlled processes. LdpA copurifes with at least KaiA and CikA, suggesting physical proximity in the cell (Ivleva *et al.*, 2005). However, yeast 2-hybrid assays do not reveal a direct connection between CikA and any of the Kai

proteins (S.R. Mackey, J.-S. Choi, and S.S. Golden, manuscript in preparation). Therefore, CikA is likely connected indirectly with these proteins as part of a larger complex. Based on the co-purification data and the model presented here, we predict that the Kai proteins and LdpA will be similarly localized in the cell to CikA.

EXPERIMENTAL PROCEDURES

Bacterial strains, culture conditions, and bioluminescence assays

The WT cyanobacterial strain *S. elongatus* PCC 7942 and its derivatives were propagated at 30º C in BG-11 medium under a photosynthetic photon flux density of 150 µmole m^{-2} s^{-1}. Antibiotics were added as needed for selection as reported previously (Bustos and Golden, 1991), except that Spectinomycin (Sp) was used at 6 µg/ml. The WT strain was transformed with *kaiB::luxAB* (integrated at neutral site II, NS2, pAM2857, Km^r) and *psbAI::luxCDE* (integrated at NS2.2, pAM1850, Cm^r) as the WT reporter strain (AMC1004) for this study (Mutsuda *et al.*, 2003). AMC1004 emits bioluminescence as a function of expression from the *kaiBC* promoter, with the *luxCDE* genes directing synthesis of the long-chain aldehyde substrate of LuxAB. The *cikA* null strain (AMC1005) was constructed by transformation of AMC1004 with pAM2152 (*cikA* insertional mutation, Gm^r). All *cikA* alleles were generated by PCR as previously described (Mutsuda *et al.*, 2003) and introduced for ectopic expression from neutral site I (NSI) in the chromosome using pAM2428 (Sp^r); this NSI vector carries an IPTG-

inducible P*trc* promoter, the *lac* operator, and *lacI* for regulated expression of cloned alleles. AMC1004 and AMC1005 were transformed with the following plasmids that encode the indicated CikA variant: pAM2477 (H-CikA), pAM2936 (H-CikA*), pAM3389 (TH-CikA), pAM3390 (TH-CikA*), pAM3391 (TH-CikAΔP), pAM3392 (TH-CikAΔN), pAM3393 (TH-CikAΔG), pAM3394 (H-PsR), pAM3619 (TH-CikA*ΔP). For expression of the fluorescent protein ZsGreen, its open reading frame (ORF) *zsg* was amplified from the plasmid pZsGreen1-N1 (BD Clontech) and cloned immediately after the P*trc* promoter in a Gateway pDonr (Clontech) NS1 targeting vector (pAM3110), creating pAM3516. The strain transformed by this plasmid was used as a control to view the subcellular localization of unfused ZsG. Alleles that encode TH-CikA and TH-CikAΔP fused to the C-terminus of ZsG were constructed with a 7-aa flexible linker (GSGSGSG) in between. These constructs were introduced into NS1 of AMC1005 by transformation with pAM3645 (TH-CikA) or pAM3646 (TH-CikAΔP). Immunoblot analysis with anti-CikA antiserum confirmed that all variants except H-PsR are expressed in the cyanobacterium and accumulate to similar levels; H-PsR was not recognized by the antiserum under the tested conditions.

Escherichia coli strains DH10B and BL21(DE3) were the hosts for plasmid propagation and protein purification, respectively. They were cultured in LB medium with the appropriate antibiotics at concentrations described previously (Mutsuda *et al.*, 2003).

Assays of circadian rhythmicity

Bioluminescence assays were performed using a Packard TopCount scintillation counter (PerkinElmer) as described previously (Mutsuda *et al.*, 2003). Data were analyzed using I&A with FFT-NLLS (available from S.A. Kay, the Scripps Research Institute, La Jolla CA, http://www.scripps.edu/cb/kay/ianda/features.htm); (Plautz *et al.*, 1997) and BRASS (available from A. Millar and P. Brown, University of Edinburgh, Edinburgh UK, http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm) software packages to calculate circadian period and relative phase. Phase-resetting phenotypes were assessed by the following protocol. Cyanobacterial strains were synchronized with two cycles of 12 h light: 12 h dark, then exposed to continuous light. At time points 3, 8, and 11 h after the onset of the next light phase, a 5-h dark pulse was given and bioluminescence was monitored in LL. The change in hours in the timing of the next circadian peak relative to an unpulsed control was calculated and plotted.

Visualization of intracellular localization of ZsGreen

Uninduced cultures that carry ZsG-TH-CikA complement the null phenotype of AMC1005, indicating function of the fusion protein under those conditions. Uninduced samples were compared to induced samples visually, and no change in localization was detected after IPTG induction. To facilitate imaging, ZsG proteins were induced by adding a final concentration of 1 mM of IPTG to fresh cultures of reporter strains and fluorescence was observed about 10 h later.

Samples (2 μl) were directly loaded on a slide treated with poly-L-lysine, covered with a coverslip and sealed with nail polish. Differential Interference Contrast (DIC) images were acquired with a Zeiss Axioplan2 microscope and Hamamatsu C5810 3CCD camera. Exposure time was 1 s for fluorescence images and 0.33 s for DIC. Red and green fluorescence were acquired separately using standard filter sets for fluoroscein isothiocyanate and Texas Red, respectively, and the images were merged. All images were processed with the publicly-available program ImageJ (http://rsb.info.nih.gov/ij). A convolution filter with default settings was applied to the red autofluorescence image arising from chlorophyll to achieve apparent sectioning of the cell and reveal the peripheral localization of the photosynthetic apparatus.

CHAPTER III

CIKA BRIDGES THE INPUT SIGNALS TO CENTRAL OSCILLATOR

INTRODUCTION

In addition to genetic results, structural information can provide more details about the function of CikA at molecular level. With a protein crystal structure, we could infer relationships among different domains, specifically the interaction between PsR and HPK as raised in the model of previous chapter. Because the protein structure of plant phytochrome has not been solved, the crystal structure of CikA might be used to model the phytochrome structure once it is successfully obtained. Because histidine kinases commonly are dimers (Dutta *et al.*, 1999), CikA was predicted to possess a dimerized structure, based on its well-conserved HPK domain. In this study, crystallization of CikA was attempted unsuccessfully; however, the purification procedures yielded useful data, including experimental demonstration that the active form of CikA in solution is a dimer. These data provide useful clues to explain how CikA is involved in circadian input pathway.

Changes in light and temperature are two main environmental cues that can bring changes in circadian rhythms because cells can be entrained or reset to the new phases through these forms of stimuli (Pittendrigh, 1981). Because CikA is found to be an input pathway component and knocking out the *cikA* gene abolishes the phase shifting ability (Schmitz *et al.*, 2000), the question is raised:

how does CikA transfer these signals to the central oscillator to reset the clock to a new phase? With a 5 h dark-pulse or temperature pulse given at different circadian time points, different phase shifting ability among WT and different *cikA* mutants may reveal the roles of each domain in the signal transduction. Previous studies showed that CikA is likely to be involved in light signal transduction (Schmitz *et al.*, 2000), cell division (Miyagishima *et al.*, 2005), redox sensing (Ivleva *et al.*, 2005) and resetting by feedback signals from overexpression of KaiC (personal communication, T. Kondo, Nagoya University). These results all suggest that CikA is likely to be a central input pathway component in the circadian system of *S. elongatus*. Thus, CikA also may be involved in transmitting another known cue to the clock, which is temperature.

The *cikA* null strains show abnormally longer cell shape than WT, which indicates the possibility that CikA is involved in cell division. In the previous chapter, I have shown that TH-CikA can complement the null phenotype in circadian rhythm, and cell shape, and that it shows a normal polar localization pattern, but TH-CikAΔP failed all assays. From these results, we concluded that the PsR domain is important for localization, and inferred that localization of CikA is likely to be important for cell division, but the functions of other domains are still not clear. Therefore, the complementation ability regarding cell division and the cellular localization of each CikA variant was examined. Combined with the circadian phenotypes of these *cikA* mutants, we can have some insight into the relationship between circadian control and cell division. According to the

model inferred from the *in vivo* results (Fig. 2-7), CikA variants with PsR, including PsR alone, are predicted to be localized at the poles just like WT.

As a supplementation, the protein expression of each CikA variant *in vivo* was checked in order to know whether the proteins are expressed properly. Protein expression level of some of them was also quantified to address their effects on their corresponding overexpression phenotypes.

RESULTS AND DISCUSSION

The active form of CikA in solution is a dimer

Recombinant H-CikA purified from *E. coli* by Ni-NTA affinity chromatography was directly used to crystallize CikA with several commonly used commercial crystallization kits. However, we failed to get CikA protein crystals, due to a serious aggregation problem. It was caused by inadequate purity of the protein sample. Thus, the protein from *E. coli* was further purified by gel filtration. As shown in Figure 3-1A, the chromatogram showed three peaks; after comparison with protein standards, it was shown that none of the peaks represents monomeric CikA. Peak 1 was close to the exclusion volume of the column, and likely includes non-specific aggregates and additional proteins, as supported by Coomassie-stained SDS-PAGE (Fig. 3-1B). Peak 3 was the purest, and assumed to be the main form of CikA in solution because: peak 1 decreased with a correspondent increase of peak 3 when DTT was added to

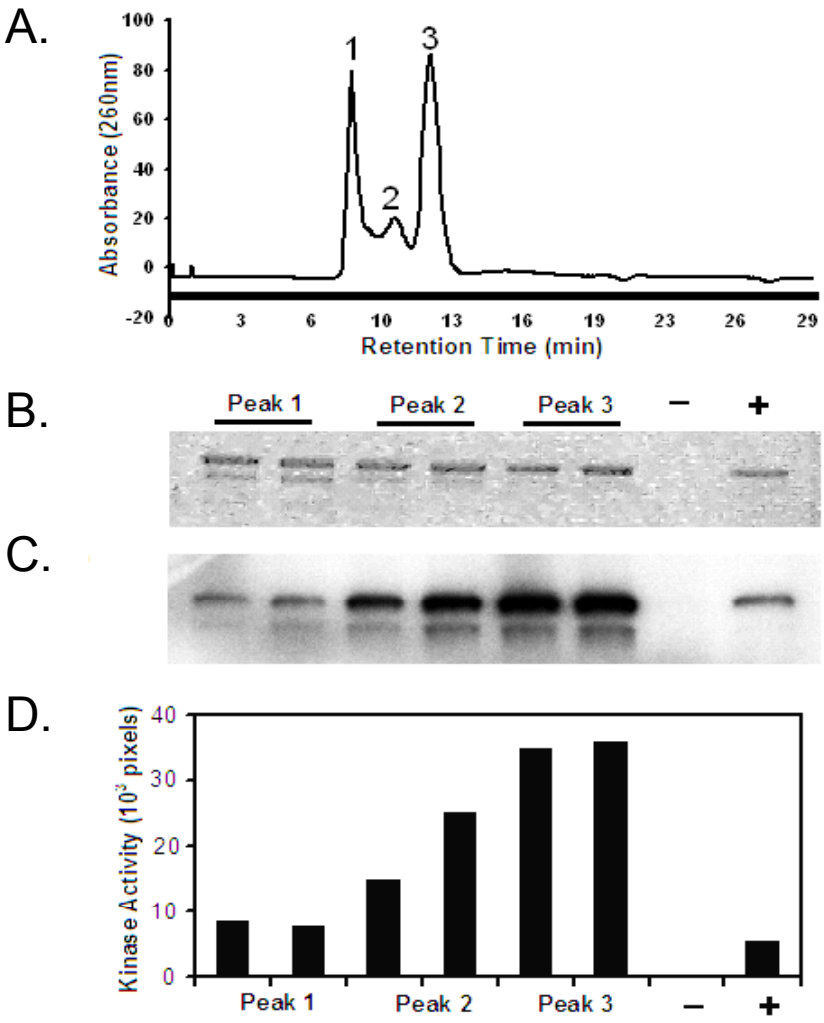


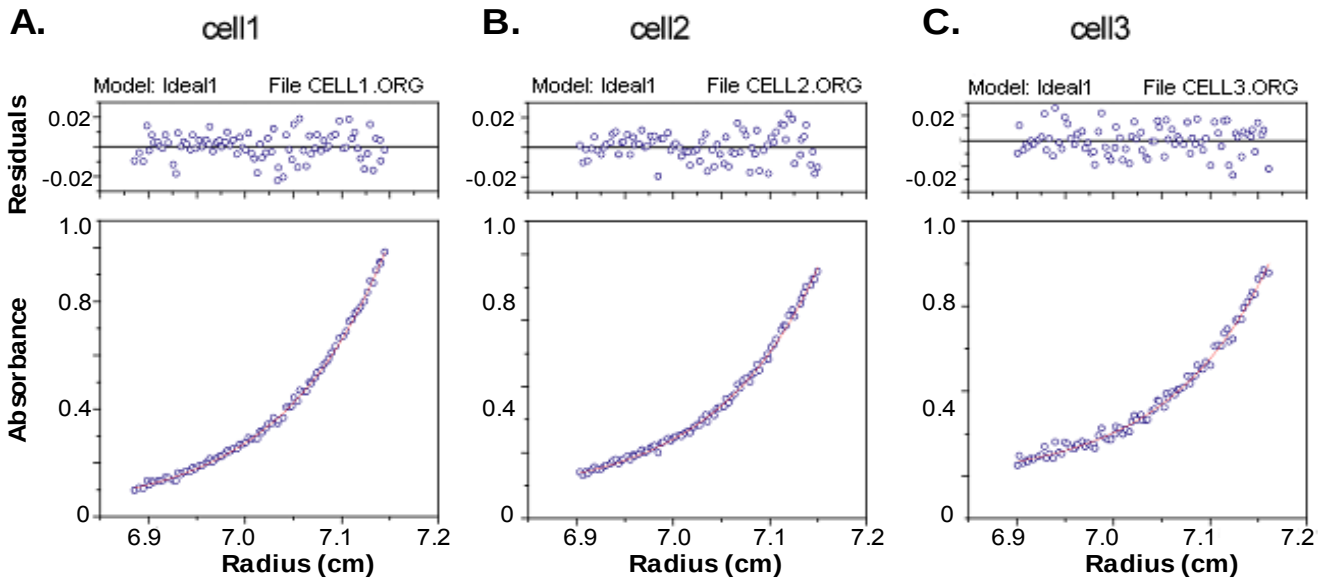
Figure 3-1 Purification of active CikA.

(A) His-tagged CikA isolated from *E. coli* by affinity chromatography was further purified by gel filtration.

(B) Two samples collected from each peak fraction were loaded on 12.5% SDS-PAGE and visualized by Coomassie Brilliant Blue to check the mobility, purity, and protein concentration (approximately 200 ng/per lane, except the positive control, approximately 100 ng/per lane).

(C) Phosphor imaging visualization of autophosphorylation of each CikA peak sample by [?-32P] ATP.

(D) Graphical representation of relative autophosphorylation activity. Imaging system: Kodak digital science1D V2.0.



Data: data1_abs
Model: ideal1
Chi^2=8.13817E-5
Co 0.095882 0.003565
H 3.911518E-6 ****
M 1.6304E5 2392
Xo 6.8860 ****
Baseline 0.011299 0.005520

Data: data1_abs
Model: ideal1
Chi^2=8.50953E-5
Co 0.074151 0.003967
H 3.911518E-6 ****
M 1.6564E5 3584
Xo 6.9040 ****
Baseline 0.064433 0.005956

Data: data1_abs
Model: ideal1
Chi^2=1.23519E-4
Co 0.045424 0.003923
H 3.911518E-6 ****
M 1.6922E5 5615
Xo 6.9010 ****
Baseline 0.011299 0.005520

Figure 3-2 Active CikA in solution is a dimer.

Protein sample collected from peak 1 was analyzed by sedimentation equilibrium to determine the oligomeric status of CikA. Protein conc.: (A) Cell 1: 0.40 mg/ml, (B) Cell 2: 0.30 mg/ml, (C) Cell 3: 0.20 mg/ml. Fitting model used to process the data: ideal1. Three replicates reported a molecular weight approximately twice that predicted for the CikA monomer (85.9 kDa) (A) Cell 1: MW=1.634 E5, X^2=8.138 E-5; (B) Cell 2: MW=1.666 E5, X^2=8.509 E-5; (C) Cell 3: MW=1.692 E5, X^2=1.235 E-4. Average MW = 1.661 E5 kDa. Thus, CikA is a dimer.

reduce aggregation; and, autophosphorylation assays showed the highest specific activity from peak 3 (Figure 3-1CD). Based on the retention time of each protein, we can estimate the size of the sample from each peak. However, the apparent size is influenced not only by the molecular weight (MW) but also by the molecular shape. The MW of the sample from peak 3 was estimated to be a tetramer (~350 kd), which is larger than the minimum molecular size (>=300 kd) that could be visualized by electron microscopy (EM). However, failure to see single molecules of CikA by EM suggested the possibility that the size of CikA may be overestimated through the gel filtration method. Analytical ultracentrifugation was chosen to determine the true MW of the active form of CikA in solution because the MW estimated from this method is not affected by factors other than size. The data showed that peak 3 contains dimeric CikA as is the status of other kinases of this class (Fig. 3-2). Comparing the estimated MW from these two methods, CikA is predicted to have a long strip shape rather than that of a globular protein. According to the genetic & bioinformatics results, and literature precedents, both the GAF and the dimerization subdomain in the HPK may contribute to the dimerization of CikA.

Structural information of PsR revealed by NMR

Because of the failure to obtain protein crystals from full-length CikA (even after additional purification), several fragments obtained after trypsin digestion were also tried for crystallography, but unfortunately all of them failed. Among these fragments, a C-terminal fragment of CikA (residues 582-754) that

contained the PsR domain (residues 629-744) was found to be soluble. Genetic results indicate that PsR is important in regulating the function of CikA mainly through interaction in the circadian input pathway. Therefore, the structure of PsR was studied by nuclear magnetic resonance (NMR). ^{15}NHSQC spectra of a series of amino-terminal truncations of this polypeptide were collected and used to optimize the domain length of PsR for NMR structure determination. We found that the domain consisting of residues 629-744 is independently folded, PsR is a monomer, and its chemical shifts are well dispersed (Figure 3-3). Results from NMR indicated the presence of four β-strands and five α-helices. The sequence of PsR shows similarities to the *bona fide* receiver domains of response regulators (RRs) in bacterial two-component systems (40% similar to CheY and NtrC, 49% similar to PhoB). Thus, it is predicted that PsR probably has a similar interaction with the DHp domain of HPK like a *bona fide* receiver protein, and represses the activity of HPK and blocks phosphoryl transfer.

Phase shift response to a 5-h pulse of darkness and high temperature pulse

Previous studies had shown that the *cikA* null strain is severely attenuated in resetting phase in response to a 5-h dark pulse. However, how CikA participates in this signal transduction through its domains is not clear. To clarify the contribution of each domain in effecting a phase shift, different CikA mutant

CikA *pseudo*-receiver domain ^{1}H-^{15}N HSQC spectrum

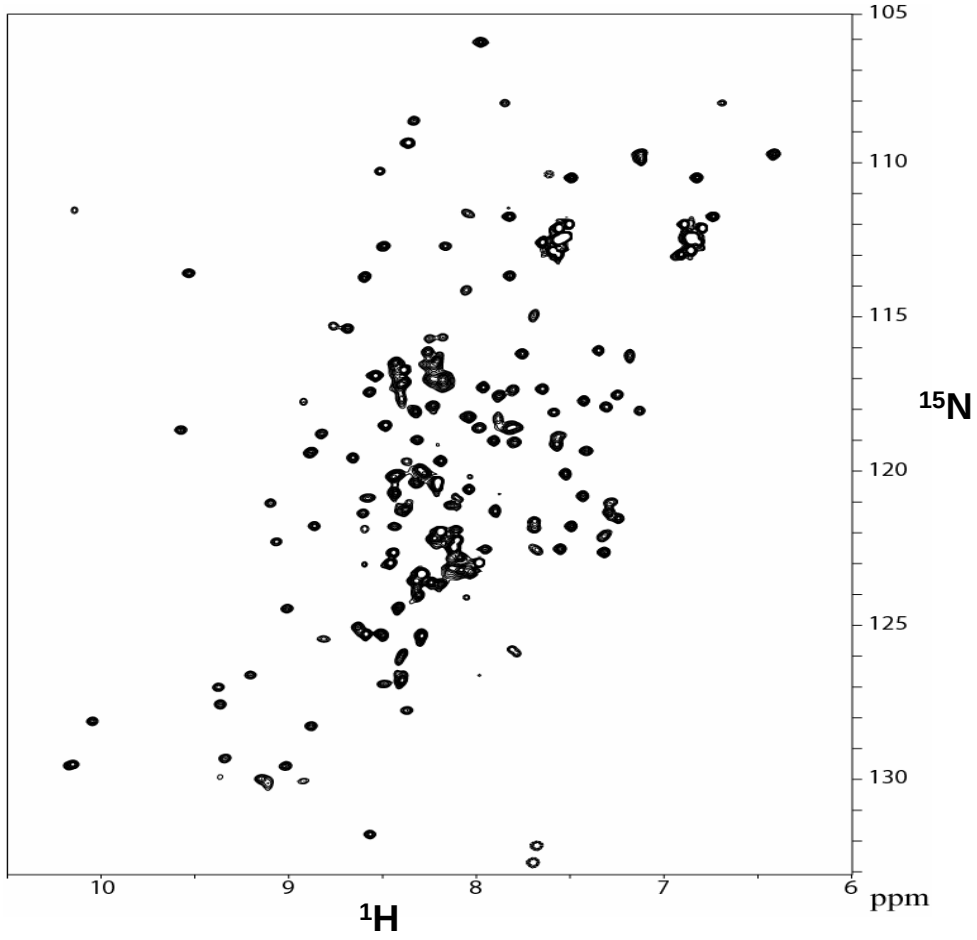


Figure 3-3 2D HSQC NMR spectrum of the *pseudo*-receiver domain (PsR) of CikA in solution.

The PsR domain protein used was expressed from a pET28-RR construct. The experimental conditions were 50 mM Sodium Chloride, 20 mM Sodium Phosphate pH 7.0, 0.5 mM protein (15N enriched), 25 ^{o}C, 14.1 T (600 MHz). Line width considerations indicated that the domain is monomeric even at the high concentration used for NMR. Spectral resolution indicates that the domain is suitable for structure determination by this method. Adapted from Gao et al., 2006.

strains were checked with a 5-h dark pulse at 3, 8 or 11 h after cells were released into the light after LD cycles (Figure 3-4). TH-CikA*, full length but without kinase activity, could not restore the phase-shifting ability of *cikA* null AMC1005 to the level of WT AMC1004, which suggests that kinase activity of HPK is directly involved in phase response. Just like TH-CikA, TH-CikAΔN could restore the phase response ability of the *cikA* null strain, as well as the circadian period and amplitude, which further showed that the N terminus is dispensable in the circadian input pathway. TH-CikAΔP, although with higher kinase activity than TH-CikA, also failed the phase shift assay; we propose that this is because it cannot localize at the poles in the absence of PsR. TH-CikAΔG and PsR, like TH-CikA*, cannot complement the period and amplitude phenotypes of a *cikA* null and also failed in phase response. Thus, kinase activity and cellular localization of CikA are likely two main functions that transfer the phase information to the central oscillator.

CikA has been found to be related with different processes, such as cell division and redox (Ivleva *et al.*, 2005; Miyagishima *et al.*, 2005). We tested whether it is also involved in temperature signal transduction. It was already known that the strains (WT or the *cikA* null strain) can be synchronized with two cycles of 12 h 30°C:12 h 40°C, as well as with light-dark cycle (data not shown), which is consistent with what we know from other organisms. However, whether CikA can affect the phase shift to the temperature pulse is still not clear. From our preliminary data, the WT and the *cikA* null strains showed different phase

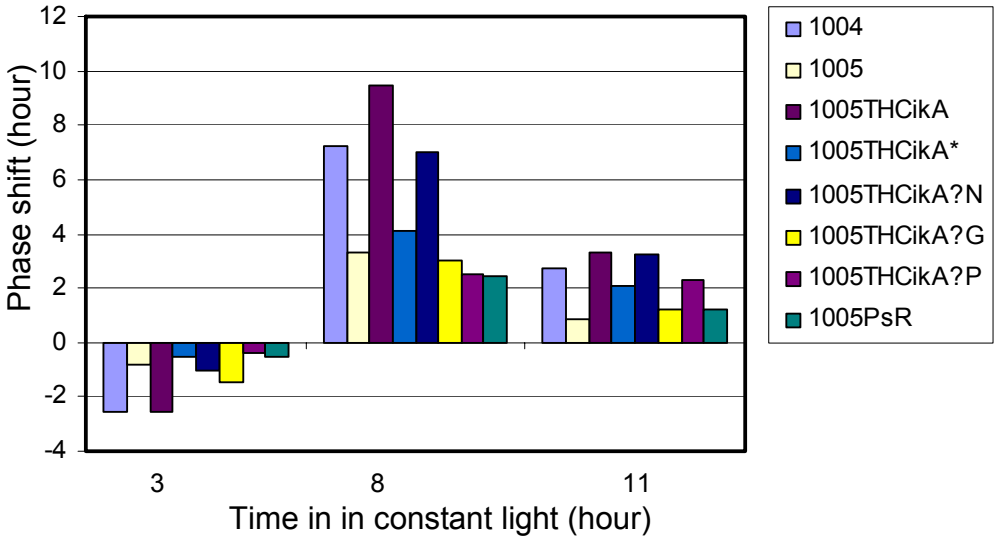


Figure 3-4 Phase shift to 5-h dark pulse of strains that express different CikA variants.

The strains were synchronized with 12 h light: 12 h dark cycle, and then exposed to constant light. At 3, 8 and 11 h after release into the light, a 5-h dark pulse (abscissa) was given. Bioluminescence was monitored for 10 days and the phase of the rhythm after the dakrk pulse was compared to that before the pulse. Positive an negative value on the ordinate indicate phase delaays and advances, respectively.

shift to the 5-h 40ºC pulse using the protocol shown in Figure 3-5, which suggested the role of CikA in this response. However, results were variable in different trials and not reported here. A more reliable assay is required to determine the function of CikA in temperature signal transduction.

Defect in cell division and cellular localization of CikA variants

Cell division is one of the physiological processes in which CikA is involved. Without CikA, the null strains showed abnormally longer cell shape than WT, and work from others indicated that knocking out the *cikA* gene affects the formation of the Z-ring (Miyagishima *et al.*, 2005). TH-CikA and TH-CikAΔN can complement the cell shape phenotype (Fig. 3-6C&E) but other CikA variants (Fig. 3-6FGH), which cannot complement the *cikA* null strains in circadian rhythms, also cannot complement the defect in cell division. This correlation suggests that the normal function of CikA in the circadian system is also important for normal function in cell division. In fresh medium, the cell shape of WT and the *cikA* null strains are similar. Only when the culture reaches the stationary phase do they begin to show differences in length. At the very late stationary phase, both WT and the null strains show an elongated cell phenotype. The reason for the different length between WT and the *cikA* null strains during the stationary phase is predicted to be caused by the changing pH and the varied luminescence intensity in the center of the culture. Because these changing conditions brought stress on the cell culture and the *cikA* null strains showed defects in Z-ring formation (Miyagishima *et al.*, 2005), the cell division for the *cikA* null strains is

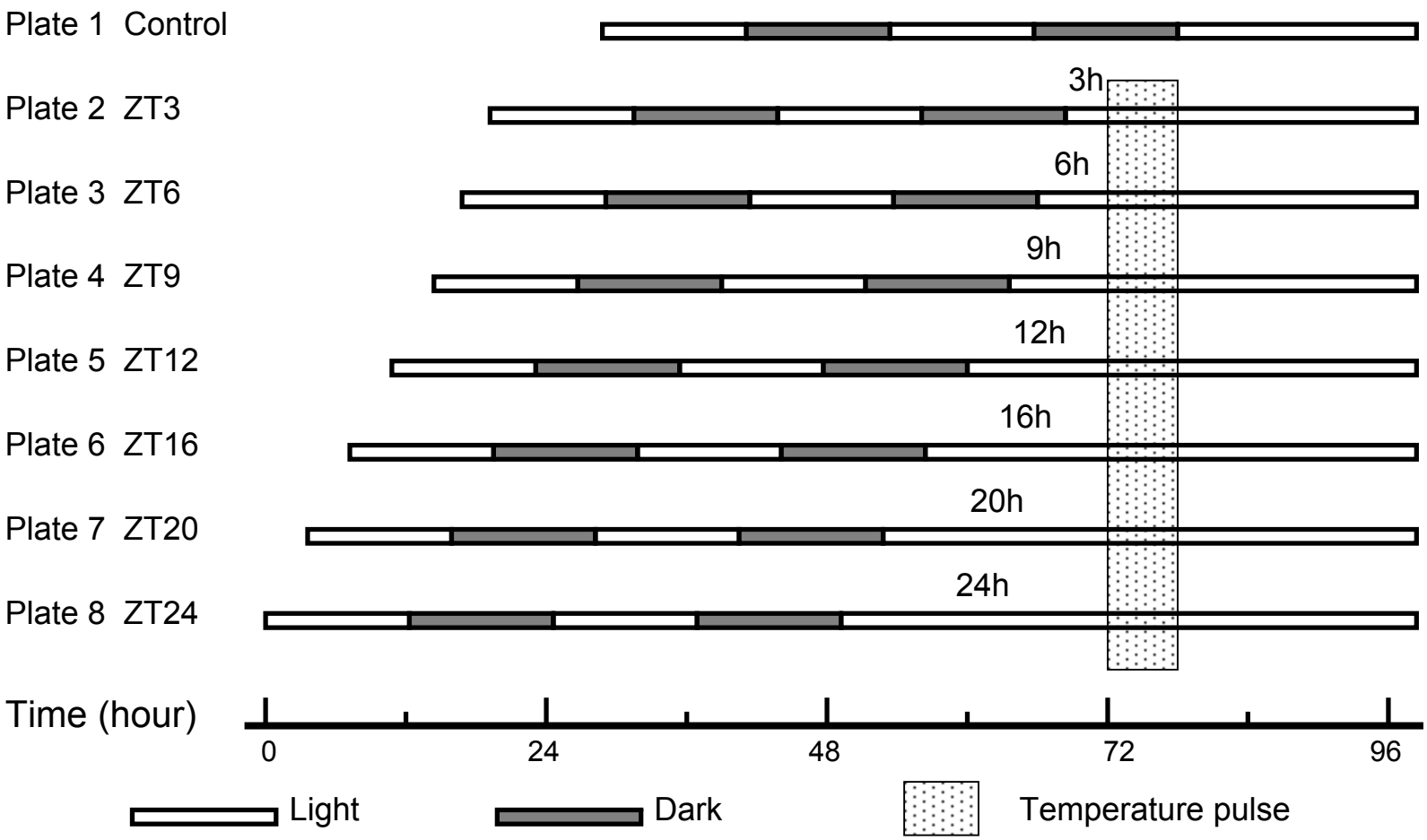


Figure 3-5 Protocol for phase shift to 5-h temperature pulse experiment.
Each plate (96 wells) carried identical samples of various genotypes with respect to the CikA variants being expressed. Each plate was exposed to LD cycles as indicated, so that they would be entrained to different phase. Plates 2-8 were administrated a 5-h temp pulse (40□C) at the same local time, which fell at a different ZT time for each plate.

more affected than that of the WT, and therefore, the cells showed abnormally long shape.

We also examined the localization of different CikA variants besides TH-CikA and TH-CikAΔP. It was predicted that PsR alone and other PsR-containing variants would also show polar localization. As predicted from the model (Figure 2-7), ZsG-TH-CikA*, ZsG-TH-CikAΔN, and ZsG-TH-CikAΔG all localize at the poles, and all of them function just like their corresponding versions without a ZsG tag (Figure 3-7ABC). However, ZsG-PsR failed to localize at the poles and showed an unevenly distributed pattern (Fig. 3-7D). Overexpression of ZsG-PsR in WT strains causes no change to the circadian rhythms (data not shown). The lack of effect on circadian rhythmicity of the ZsG construct, when overexpression of H-PsR is known to dramatically affect circadian period (Fig. 2-4), suggested that the ZsG-PsR construct is not biologically active, and its localization is not relevant. The failure was ascribed to structural differences between ZsG-PsR and PsR. Because PsR is much smaller (12 Kd) than ZsG (30 Kd), and ZsG is a tetramer and tends to aggregate in the cells, PsR-specified localization signals in the PsR domain of ZsG-PsR may be unduly influenced by the large tetramerized ZsG domain. Thus, ZsG would fail to dock into the polar position. Comparing TH-CikAΔP with TH-CikA and TH-CikAΔN, we concluded that kinase activity cannot fulfill signal transduction without docking into the right position at the poles. Thus, we propose that normal phosphoryl transfer from CikA to CikR likely occurs after localization.

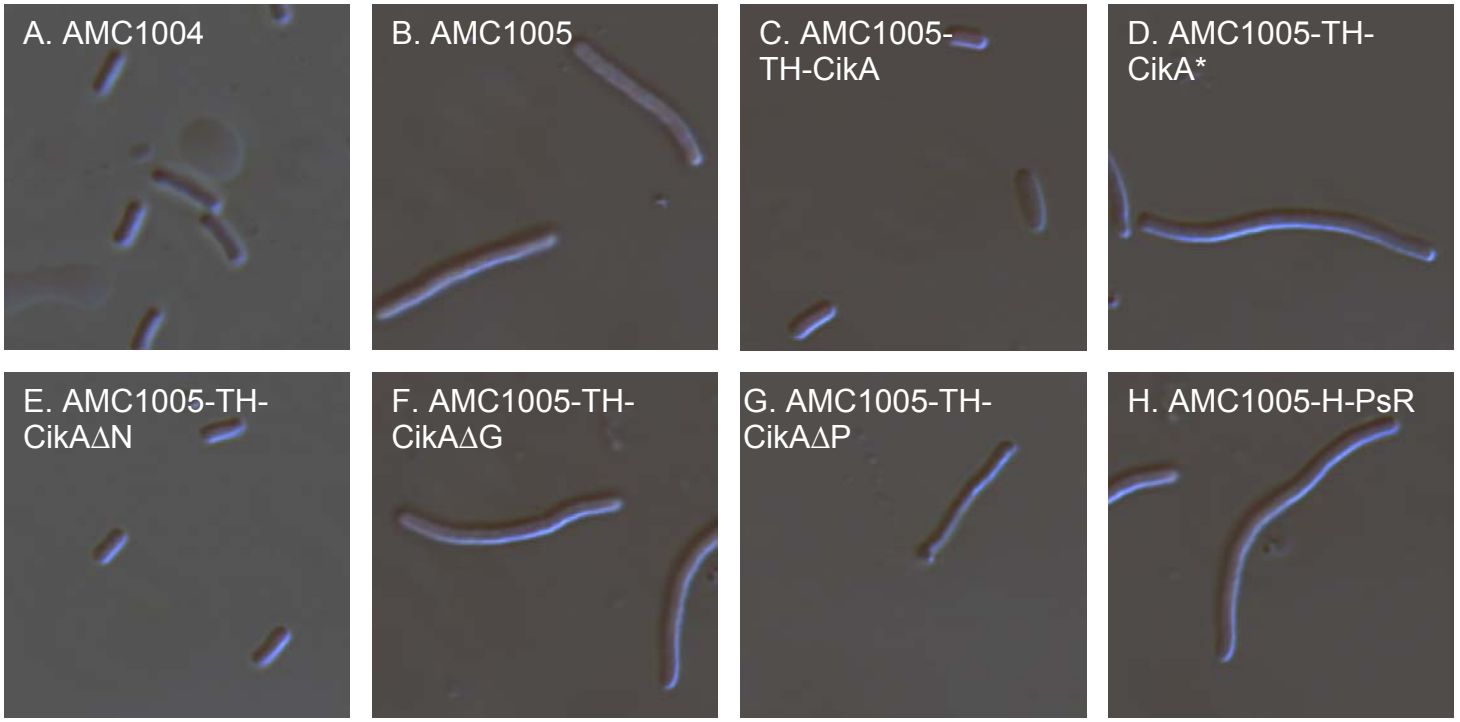


Figure 3-6 Disruption of the *cikA* gene affects cell division.

DIC images are shown for the following strains: (A) AMC1004, WT strains, (B) AMC1005, *cikA* null, (C) AMC1005 expressing TH-CikA, (D) AMC1005 expressing TH-CikA*, (E) AMC1005 expressing TH-CikAΔN, (F) AMC1005 expressing TH-CikAΔG,(G) AMC1005 expressing TH-CikAΔP, and (H) AMC1005 expressing H-PsR.

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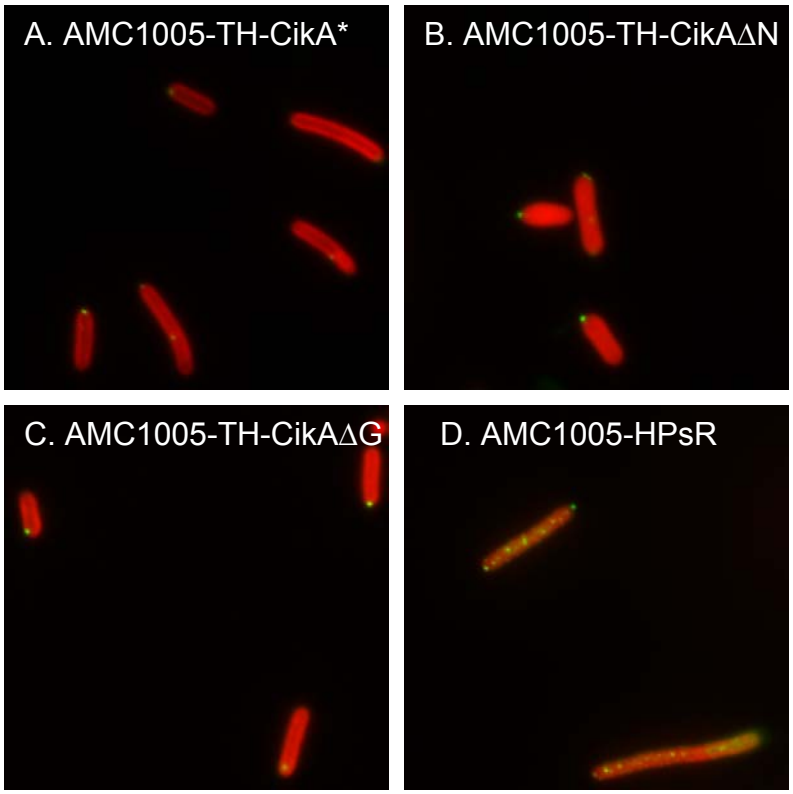

Figure 3-7 Cellular localization of several CikA variants.
CikA variants that include PsR show polar localization, but PsR alone failed to show this pattern. Fluorescence images are shown for AMC1005 transformed with ectopically-expressed alleles that encode: ZsG-TH-CikA* (panel A); ZsG-TH-CikAΔN (panel B); ZsG-TH-CikAΔG (panel C); ZsG-TH-CikAΔN (panel D). Red fluorescence arises from autofluorescence of chlorophyll in the photosynthetic apparatus and marks the cell periphery where thylakoid membranes are located. Expression of the ZsG-encoding alleles was induced with 100 μM IPTG to facilitate imaging; the same localization was observed by eye without induction in each case.

Protein expression of different CikA variants

From the complementation test and overexpression phenotypes of different CikA variants, we expect that these proteins function normally as expected. As supplementary information, we wanted to know whether these proteins are properly expressed and what is the expression level in the cells. These data would ensure that conclusion based on qualitative differences among constructs that carry different domains were not actually due to quantitative differences in expression or stability. As the Western blots results shown in Figure 3-8A, all the CikA variants express properly with the expected size. However, we were not able to detect PsR alone with the anti-CikA antiserum. Another member of the lab has detected PsR on the Western blots from the same construct I used; at the time of writing, I have not identified the problem with my western blot procedure.

A titration assay was performed to distinguish the overexpression phenotype of TH-CikA and TH-CikA* and that of TH-CikAΔP and TH-CikA*ΔP (Figure 2-5). In order to address the relationship between protein expression level at different IPTG concentration and their corresponding overexpression phenotypes, TH-CikA and TH-CikAΔP were selected to determine the protein expression levels (Figure 3-8B&D, C&E). Without induction, the protein expression level of both strains is roughly the same as that of the positive control (native WT CikA). At 7.5 μM IPTG, where these two strains begin to show different amplitudes than the WT, the protein expression level is almost same. Above 10 μM IPTG, the

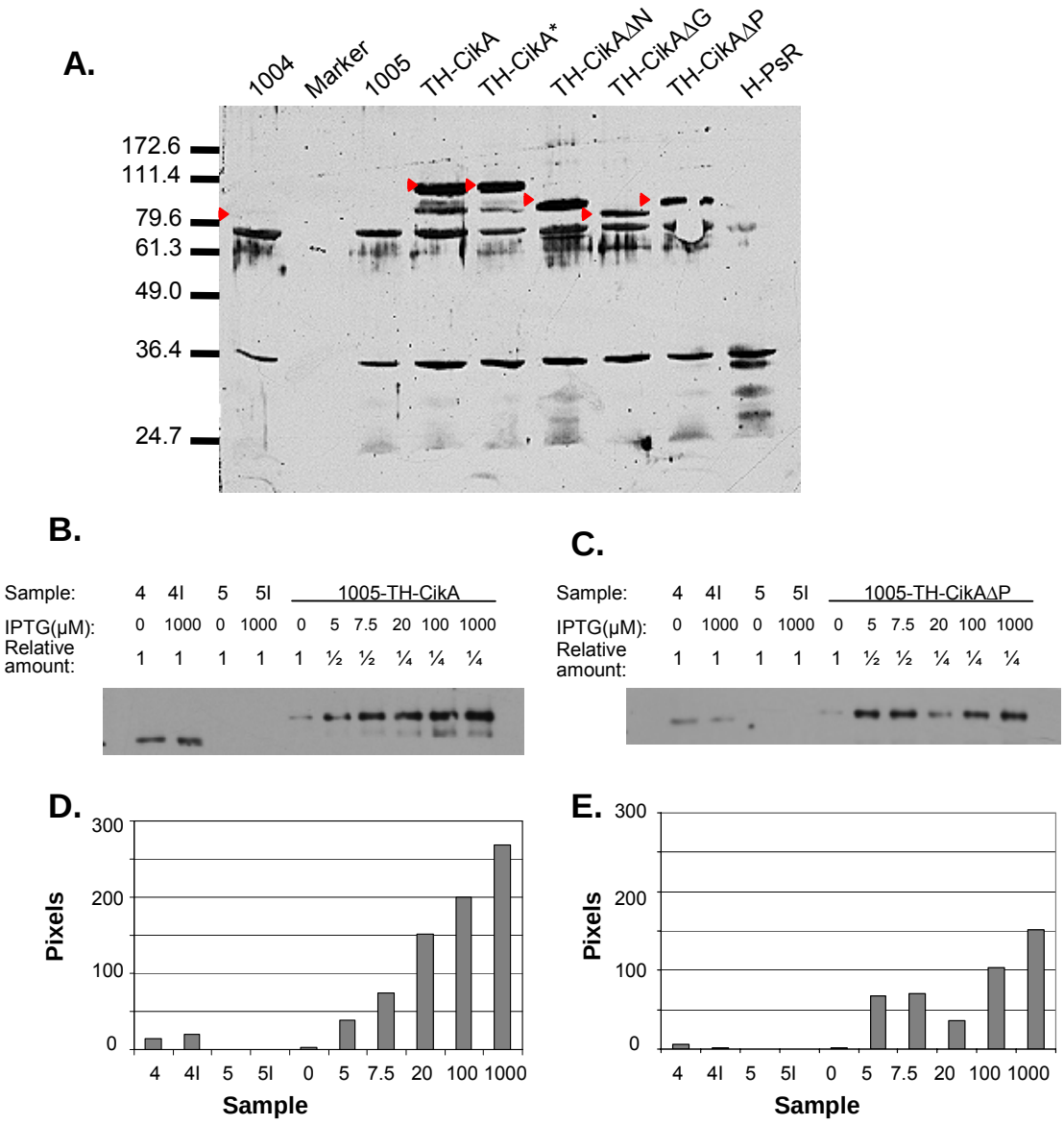

Figure 3-8 Protein expression levels of each different CikA variants.

(A) Western Blot for different CikA variants, about 7 μg total protein from cyanobacteria loaded for each lane, and CikA anti-serum from rabbit was used. The expected bands were marked with red arrow. The CikA band in AMC1004 is much weaker than other samples, which were induced by addition of 1 mM IPTG for 5 h and collected. Expression levels of TH-CikA (B) and TH-CikAΔP (C) at the different IPTG concentrations. Graphical representation of these expression levels is shown in (D) and (E) respectively, which are normalized to the same amount of protein. The lane labeled with relative amount of 1 was loaded with about 3 μg total protein. Values of ½ or ¼ mean only half (1.5 μg) or one quarter (0.75 μg) of total protein loaded compared with the lane with relative amount of 1. For the samples: 4 – AMC1004 without induction; 4I– AMC1004 with 1 mM IPTG induction; 5—AMC1005 without induction; 5I-AMC1005 with 1 mM IPTG induction.

overexpression phenotype of TH-CikA and TH-CikA* became arrhythmic (Fig. 2-5H).

EXPERIMENTAL PROCEDURES

Bacterial strains, culture conditions, and protein purification

All strains, plasmids, and primers used in this study are listed in Tables 3-1 and 3-2. The construction methods for these strains and plasmids, culture conditions, and the protein purification method are described in Chapter II.

Gel-filtration

The 6His tagged CikA purified from *E. coli* using Ni-NTA affinity matrix (Qiagen, Valencia, CA), was further purified by gel-filtration method. An AKTA FPLC system (Amersham Life Science) was used for this study; a column of Superdex-200 was first used to characterize the sample, then a column of Sephacryl S300 was used for large volume sample preparation; the elution buffer includes 20 mM HEPES, 150 mM NaCl, 5 mM DTT, and 1 mM EDTA. The elution speed is 1 ml/min for Superdex-200 and 0.5ml/min for Sephacryl S300; elution time is 30 min and 3 h for each column respectively; and the eluted fractions were monitored with a UV detector at 260 nm.

Crystallization method

Initial crystallization conditions were screened using Crystal Screen 1 & 2 (Hampton Research). Crystals were grown using the hanging drop vapor diffusion method at 16°C (Cho *et al.*, 2003). By trypsin digestion, several soluble

Table 3-1 Strains and plasmids used to study the function of CikA in *S. elongatus* PCC 7942

Strains and Primers	Plasmids for transformations	Marker	Characteristics of plasmids and strains	Source
E. coli				
DH10B			Host for plasmids	Invitrogen[a]
BL21(DE3)		Km	Host for protein overexpression	Novagen[b]
AM2428	pAM2428	Sp	DH10B contains pAM2428 to do NSI transformation. NSI vector, contains Ptrc promoter, N terminal must use *SmaI*	Lab Collection
AM0027	pUC19	Ap	Contains pUC19	UC collection[c]
AM2477	pAM2477	Sp	pAM2428 with *Ptrc::His-cikA*	Lab Collection
AM3341	pAM3341	Gm	pUC19-CikA-UGD, cikA deletion plasmid.	This study
AM3389	pAM3389	Sp	*Ptrc::Trx-His-cikA*; PCR product (AMO1505-AMO1506) from pAM2932, cut with *SmaI-HindIII* and cloned into pAM2428.	This study
AM3390	pAM3390	Sp	*Ptrc::Trx-His-cikA**; PCR product (AMO1505-AMO1506) from pAM2937, cut with *SmaI-HindIII* and cloned into pAM2428.	This study
AM3391	pAM3391	Sp	*Ptrc::Trx-His-cikA-ΔPsR*; PCR product (AMO1505-AMO1506) from pAM2760, cut with SmaI-HindIII and cloned into pAM2428.	This study
AM3392	pAM3392	Sp	*Ptrc::Trx-His-cikA-ΔN*; PCR product (AMO1505-AMO1506) from pAM2933, cut with *SmaI-HindIII* and cloned into pAM2428.	This study
AM3393	pAM3393	Sp	*Ptrc::Trx-His-cikA-ΔGAF*; PCR product (AMO1505-AMO1506) from pAM2934, cut with SmaI-HindIII and cloned into pAM2428.	This study
AM3394	pAM3394	Sp	*Ptrc::His-PsR*; PCR product (AMO1505-AMO1506) from pAM2761, cut with *SmaI-HindIII* and cloned into pAM2428.	This study
AM3619	pAM3619	Sp	*Ptrc::trx-his-cikA*-ΔPsR*; fragment cut from pAM3390 with *ApaI/AvrII* and cloned into pAM3391.	This study
AM3516	pAM3516	Sp	*Ptrc::ZsG*, used as a control.	Lab Collection
AM3645	pAM3645	Sp	*Ptrc::ZsG-Trx-His-cikA*; Combined PCR product with PCR fragment (AMO1507-AMO1508) from pAM2932 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study
AM3646	pAM3646	Sp	*Ptrc::ZsG-Trx-His-cikA-ΔPsR*; Combined PCR product with PCR fragment (AMO1507-AMO1509) from pAM2760 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study
AM3732	pAM3732	Sp	*Ptrc::ZsG-Trx-His-cikA-ΔN*; Combined PCR product with PCR fragment (AMO1507-AMO1508) from pAM2933 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study
AM3733	pAM3733	Sp	*Ptrc::ZsG-Trx-His-cikA-ΔG;* Combined PCR product with PCR fragment (AMO1507-AMO1508) from pAM2934 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study
AM3734	pAM3734	Sp	*Ptrc::ZsG-Trx-His-cikA**; Combined PCR product with PCR fragment (AMO1507-AMO1508) from pAM2937 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study
AM3739	pAM3739	Sp	*Ptrc::ZsG-His-PsR*; Combined PCR product with PCR fragment (AMO1510-AMO1511) from pAM2761 and PCR fragment (AMO1503-AMO1504) from pAM3525, BP cloning into pAM3110.	This study

Table 3-1 continued

Strains and Primers	Plasmids for transformations	Marker	Characteristics of plasmids and strains	Source
S. elongatus				
AMC1004	pAM1850, pAM2857	Km, Cm	*KaiB*::*luxAB* fusion in NSII and *psbAI*::*luxCDE* fusion in NS2.2	This study
AMC1005	pAM1850, pAM2857, pAM2152	Km, Gm, Cm	Constructed from AMC1004 by knocking out *cikA* using pAM2477	This study
AMC1305	pAM1850, pAM2857, pAM3341	Km, Gm, Cm	Constructed from AMC1004 by deletion of *cikA* using pAM3341	This study
AMC1006	pAM2477	Km, Gm, Cm, Sp	AMC1005 complemented with CikA fusion in NSI	This study
AMC1322	pAM3389	Km, Cm, Sp	AMC1004 transformed with pAM3389 (Trx-His-CikA)	This study
AMC1323	pAM3390	Km, Cm, Sp	AMC1004 transformed with pAM3390 (Trx-His-CikA*)	This study
AMC1324	pAM3391	Km, Cm, Sp	AMC1004 transformed with pAM3391 (Trx-His-CikAΔP)	This study
AMC1325	pAM3392	Km, Cm, Sp	AMC1004 transformed with pAM3392 (Trx-His-CikAΔN)	This study
AMC1326	pAM3393	Km, Cm, Sp	AMC1004 transformed with pAM3393 (Trx-His-CikAΔG)	This study
AMC1327	pAM3394	Km, Cm, Sp	AMC1004 transformed with pAM3394 (Trx-His-RR)	This study
AMC1328	pAM3390	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3390 (Trx-His-CikA-H393A)	This study
AMC1329	pAM3391	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3391 (Trx-His-CikAΔP)	This study
AMC1330	pAM3392	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3392 (Trx-His-CikAΔN)	This study
AMC1331	pAM3393	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3393 (Trx-His-CikAΔG)	This study
AMC1332	pAM3394	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3394 (Trx-His-RR)	This study
AMC1333	pAM3389	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3389 (Trx-His-CikA)	This study
AMC1334	pAM3390	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3390 (Trx-His-CikA-H393A)	This study
AMC1335	pAM3391	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3391 (Trx-His-CikAΔP)	This study
AMC1336	pAM3392	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3392 (Trx-His-CikAΔN)	This study
AMC1337	pAM3393	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3393 (Trx-His-CikAΔG)	This study
AMC1338	pAM3394	Km, Gm, Cm, Sp	AMC1305 transformed with pAM3394 (Trx-His-PsR)	This study
AMC1497	pAM3619	Km, Cm, Sp	AMC1004 transformed with pAM3619 (Trx-His-CikA*ΔP)	This study
AMC1498	pAM3619	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3619 (Trx-His-CikA*ΔP)	This study
AMC1437	pAM3516	Km, Cm, Sp	AMC1004 transformed with pAM3516 (ZsG only)	This study
AMC1438	pAM3516	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3516 (ZsG only)	This study
AMC1420	pAM3645	Km Gm, Cm, Sp	AMC1005 transformed with pAM3645 (ZsG-Trx-His-CikA)	This study
AMC1421	pAM3646	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3646 (ZsG-Trx-His-CikA-ΔPsR)	This study
AMC1499	pAM3732	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3732 (ZsG-Trx-His-CikA-ΔN)	This study
AMC1500	pAM3733	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3733 (ZsG-Trx-His-CikA-ΔG)	This study
AMC1501	pAM3734	Km, Gm, Cm, Sp	AMC1005 transformed with pAM3734 (ZsG-Trx-His-CikA*)	This study

[a] Invitrogen, Carlsbad, California

[b] Novagen, Madison, WI.

[c] UC-Berkerly, California

Table 3-2 Main primers used in this study

Primers	Length	Sequence 5'→ 3'	Characteristics	Source
AMO1505	32	TCC CCC GGG TGA GCG ATA AAA TTA TTC ACC TG	Forward primer, to amplify genes for different Trx tagged CikA variants from their protein expression plasmid with pET32 as vector. These fragments were cut with *SmaI/HindIII* and cloned into pAM2428. This primer contains *Sma*I site.	This study
AMO1506	27	CCG CAA GCT TGC TGC AGG TCG ACT CGC	Reverse primer, paired with AMO1505. It contains *HindIII* site.	This study
GD001	51	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC ATC CTG CGA TGC AGA TCT GGC	Forward primer to amplify *zsg* from AM3525. It carries the *attB1* site.	Lab collection
GD002	41	TCC TGA ACC CGA TCC AGA GCC GGG CAA GGC GGA GCC GGA GG	With GD002 to amplify *zsg.* It carries the *attB2* site.	Lab collection
AMO1507	45	GCC TCT GGA TCG GGT TCA GGA ATG AGC GAT AAA ATT ATT CAC CTG	Forward primer, to amplify genes of different Trx tagged cikA variants from their protein expression plasmid with pET32 as vector. Together with ZsG PCR fragment (AMO1503-AMO1504) to do a combine PCR, the products were cloned into pAM3110 by BP cloning.	This study
AMO1508	54	GGG GAC CAC TTT GTA CA GAA AGC TGG GTT AAC TGT TGC AAC ACA ACC GCT AGG	Reverse primer, paired with AMO1507 to amplify Trx tagged CikA variants with PsR domain.	This study
AMO1509	51	GGG GAC CAC TTT GTA CA GAA AGC TGG GTT ATG GAC GGT CTA GGC TCG ACG	Reverse primer, paired with AMO1507 to amplify Trx tagged CikA variants without PsR domain.	This study
AMO1510	43	GCC TCT GGA TCG GGT TCA GGA ATG GGC AGC AGC CAT CAT CAT C	Forward primer, to amplify H-PsR from its protein expression plasmid with pET32 as vector. Together with ZsG PCR fragment (AMO1503-AMO1504) to do a combine PCR, the products were cloned into pAM3110 by BP cloning.	This study
AMO1511	55	GGG GAC CAC TTT GTA CA GAA AGC TGG GTC TAG GAA CTC GGG CGA TCG CCC TCT G	Reverse primer, paired with AMO1510 to amplify H-PsR. It contains sequence for BP cloning.	This study

fragments were obtained and the sequences of their N-termini were identified by MALTI-TOF to recognize their positions in full-length CikA. According to the amino acid sequence of these soluble fragments, protein expression plasmids were constructed to prepare protein for crystallization. The PsR domain was one of these three fragments.

Sedimentation equilibrium

Analytical ultracentrifugation was performed to determine the oligomeric status of CikA using a Beckman Optima XL-A Analytical ultracentrifuge. The procedure followed “CMD XL-A Users Manual” from Beckman Instruments. A rotor of Ti60 was used with a rotating speed at 8,000 rpm for 48 hours. The solution was monitored at 260 nm. The data were fit to an ideal monodisperse model using the program Origin (Microcal Software, Inc., Northampton, MA) with the Fitting Model ideal1.

NMR

Methods are described in Gao, *et al.,* 2005.

Phase shift assays

The protocol for the phase shift to a 5 h dark pulse is described in Chapter II.

The phase-shift experiment using a temperature pulse was done as described in Figure 3-5. All the strains were synchronized with two cycles of 12 h L:12 h D and released into LL before given a 5 h temperature pulse at the time indicated. To treat sample plates with darkness, each sample plate on the TopCount was replaced with a black dummy plate and then kept in a dark box

for 12 h. To give a temperature pulse on TopCount, the cooling fan was turned off, which allowed the temperature in the sample towers, which were surrounded by a light box, to increase from 30°C to 41°C within 10 min. It is important that the unpulsed control plate was not on the TopCount machine when other plates were given a temperature pulse. It was replaced with a dummy plate, kept at 30°C in the darkness to be entrained, and returned to the TopCount tower after the entrainment was done and temperature in the sample tower was returned to 30°C.

Visualization of intracellular localization of ZsGreen

Intracellular localization of ZsGreen-fused CikA variants is described in Chapter II.

CHAPTER IV

CONCLUSIONS

As an unusual phytochrome-like protein, CikA is a key input pathway component of the circadian clock in *S. elongatus*. So far, its function is found to be related with transduction of environmental signals including light and temperature (possible), cell division, redox status of the cells, and feedback signals from central oscillator by overexpression of KaiC. How can CikA bridge these different signals to the central oscillator? Besides the kinase activity of HPK, the regulation of its unusual PsR domain compared with other bacteriophytochrome was proved to be the key to understand its function *in vivo*.

Obviously, the kinase activity of HPK is crucial to its normal function because TH-CikAHA and other variants, which kinase activity is non-detectable *in vitro*, cannot complement the *cikA* null strains in all the phenotypes that have been examined. Furthermore, although TH-CikAΔN has much lower kinase activity than WT CikA, it still can complement the null. Thus, it is likely that the phosphoryl transfer is more important than the level of kinase activity for the function of CikA, and that the phosphoryl is very likely transferred to its cognate partner like a response regulator in two-component signal transduction systems. Without the transfer of the phosphoryl, even though TH-CikAHA has the same cellular localization as TH-CikA, which indicates TH-CikAHA can normally interact with other components, it still cannot transmit the input information to the

central oscillator. It is also possible that CikR does not exist at all. The phosphorylation status may regulate the function of CikA likely through the conformational change of CikA. TH-CikAHA cannot complement the null possibly due to the abolishment of autophosphorylation ability. This possibility can be determined by introduction of an amino acid (Glu) to site 393 to mimic a phosphorylated CikA and checking its complementation ability. If this variant can complement, it will suggest that the conformational change of CikA caused by phosphorylation is important to the function of CikA instead of phosphoryl transfer. It also suggests CikR probably does not exist at all.

PsR was found to regulate the involvement of CikA in the circadian input pathway of *S. elongatus*. First, PsR regulates the kinase activity of HPK. The model predicted from the *in vitro* data, specifically that PsR negatively regulates the kinase activity of HPK, was further confirmed by the *in vivo* data. Overexpression of kinase-proficient and -deficient constructs TH-CikAΔP and TH-CikA*ΔP in WT strains produced differences in circadian amplitudes; however, the PsR-containing kinase-proficient and -deficient air did not show this distinction at any level of the induction. We conclude that the repression of HPK by PsR in full-length variants masks this difference. Since PsR shows a similar structure with a *bona fide* response regulator, it is predicted to interact with the DHp subdomain of HPK and block the position that CikR is required to dock into to receive the phosphoryl group. This mechanism can be further

shown by tests of direct protein-protein interaction between TH-CikAΔP and PsR *in vitro*.

Second, PsR likely regulates the location of the phosphoryl transfer from HPK to its cognate response regulator to transmit the input signals. CikA is found to localize at the poles. Without PsR, TH-CikAΔP fails to localize at the poles and cannot complement the null strains even though it has 10-fold stronger kinase activity than TH-CikA. Different overexpression phenotypes between TH-CikAΔP and TH-CikA*ΔP suggest a role of kinase activity in lowering down the amplitude, probably by transfer of its phosphoryl to CikR or possibly to other components that can be phosphorylated. We predict that the decrease in amplitude in TH-CikAΔP is because CikR receives a phosphoryl in cytosol instead of at the poles. With the model suggested in Figure 2-7, only after CikA localizes at the poles through the interaction of PsR with its interaction partners, the negative control of PsR to HPK is released and CikR can dock into and receive the phosphoryl and then activate the next process. However, CikR receiving a phosphoryl in cytosol is not a normal signal transduction. The data suggest that the phosphoryl transfer needs to be at the proper position to fulfill a normal signal transduction in circadian system of *S. elongatus*. It seems that CikA transmits the input signals to the central oscillator at the poles. Thus, the central oscillator is predicted to localize at the poles. Preliminary results of the cellular localization of Kai proteins likely support this prediction (unpublished data from G. Dong and S.S. Golden).

Even though no direct interaction with Kai proteins was demonstrated from the results of yeast two-hybrid assays, CikA is still likely involved in the periodosome because CikA can be copurified with LdpA and Kai proteins (Ivleva *et al.*, 2005). Another piece of evidence is the dominant arrhythmic phenotype after overexpression of CikA and other PsR-harbored variants, which suggests that the oscillator can be disturbed by the interaction of excessive CikA in the cells. Some components probably function as intermediates to bring CikA and Kai proteins together, and LdpA is one of the candidates. Other possible components are predicted to be cell division proteins because *cikA* null strains show a defect in cell division without CikA. Several protein kinases related with cell division, such as PleC, DivJ and CckA, as well as some of their response regulators, localize at the poles in *Caulobacter crescentus* (Quardokus and Brun, 2003), a model system for cell division. Thus, cell division proteins are also candidates. Another prediction is the localization of the periodosome, in which KaiC is likely to function as the core protein. If the periodosome localizes at the poles, that localization can be used to address how the circadian system times cell division to allow it to occur only during the subject daytime. Preliminary data have shown that KaiC also localizes at the poles. We predict that CikA would lose its polar localization pattern in a strain in which genes coding its interaction partners are knocked out. These genes may include Kai proteins, CikR, LdpA, or some cell division proteins.

Future experiments will be designed to identify CikR and the interaction partners either by copurification methods or by examination of the localization of CikA after the possible candidate genes are knocked out or overexpressed. The function of the featureless N terminus and GAF will also be investigated in order to understand how different signals can be interpreted and transmitted to the central oscillator.

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